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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/15, C07K 14/81, A61K 38/57 — A2

(11) International Publication Number: WO 97/33996

(43) International Publication Date: 18 September 1997 (18.09.97)

(21) International Application Number: PCT/US97/03894

(22) International Filing Date: 10 March 1997 (10.03.97)

(30) Priority Data:

60/013,106	11 March 1996 (11.03.96)	US
60/019,793	14 June 1996 (14.06.96)	US
08/725,251	4 October 1996 (04.10.96)	US

(60) Parent Applications or Grants
(63) Related by Continuation
US 08/725,251 (CIP) Filed on 4 October 1996 (04.10.96)
US 60/019,793 (CIP) Filed on 14 June 1996 (14.06.96)
US 60/013,106 (CIP) Filed on 11 March 1996 (11.03.96)

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: HUMAN BIKUNIN

(57) Abstract

The instant invention provides for proteins, polypeptides, nucleic acid sequences, constructs, expression vectors, host cells, pharmaceutical compositions of, and methods for using human placental bikunin, serine protease inhibitor domains, and fragments thereof.

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Title of the Invention: Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

Protein Serine Protease Inhibitor

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98, 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hem rrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979)Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976) , Artzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al., in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant invention embodies a protein having the amino acid sequence:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS                        179
```

(SEQ ID NO: 1)

In a prefered embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK                                  170
```

(SEQ ID NO: 52)

In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH2-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

```
AGSFLAWLGSLLLSGVLA                                          -1
ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN         50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF        100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE        150
ACMLRCFRQQENPPLPLGSKVVVLAGAVS                              179
```

(SEQ ID NO: 2)

In a prefered embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

```
MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
```

(SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

```
MLR AEADGVSRLL GSLLLSGVLA  -1
```

(SEQ ID NO: 54)

In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH2-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid sequence:

```
IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRCFRQ                                            159
```

(SEQ ID NO: 3)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```
CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRC                                               156
```

(SEQ ID NO: 50).

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATV 64
(SEQ ID NO: 4)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-61)" having the amino acid sequence:

CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKC 61
(SEQ ID NO: 5)

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC 156
(SEQIDNO:7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS 92
(SEQIDNO:8)

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

```
1)EST                          MLR AEADGVSRLL GSLLLSGVLA  -1
2)PCR                        MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
3)λcDNA                      MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

1)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
2)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
3)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

1)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
2)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
3)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

1)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
2)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
3)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

1)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
2)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
3)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

1)QERALRTVWS SGDDKEQLVK NTYVL                          225
2)QERALRTVWS FGD                                      213
3)QERALRTVWS SGDDKEQLVK NTYVL                         225
```

where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO:47, and 3) is lambda cDNA clone SEQ ID NO:49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

```
EST     VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
EST     QERALRTVWS SGDDKEQLVK NTYVL       225
```

(SEQ ID NO: 69)

a transmembrane amino acid sequence:

```
PCR     VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
PCR     QERALRTVWS FGD                    213
```

(SEQ ID NO: 68)

or a transmembrane amino acid sequence:

```
λcDNA   VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
λcDNA   QERALRTVWS SGDDKEQLVK NTYVL       225
```

(SEQ ID NO: 67).

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In an another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa^{1} through Xaa^{32} in the amino acid sequence for nativ placental bikunin. Substitutions at Xaa^{1} through Xaa^{16} are also preferred for variants of bikunin (7-64), while substitutions at Xaa^{17} through Xaa^{32} are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

Ala Asp Arg Glu Arg Ser Il Xaa[1] Asp Ph 10
Cys Leu Val Ser Lys Val Xaa[2] Gly Xaa[3] Cys 20
Xaa[4] Xaa[5] Xaa[6] Xaa[7] Xaa[8] Xaa[9] Trp Trp Tyr Asn 30
Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa[10] 40
Tyr Xaa[11] Gly Cys Xaa[12] Xaa[13] Xaa[14] Ser Asn Asn 50
Tyr Xaa[15] Thr Lys Glu Glu Cys Leu Lys Lys 60
Cys Ala Thr Xaa[16] Thr Glu Asn Ala Thr Gly 70
Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp 80
Ser Ser Val Pro Ser Ala Pro Arg Arg Gln 90
Asp Ser Glu His Asp Ser Ser Asp Met Phe 100
Asn Tyr Xaa[17] Glu Tyr Cys Thr Ala Asn Ala 110
Val Xaa[18] Gly Xaa[19] Cys Xaa[20] Xaa[21] Xaa[22] Xaa[23] Xaa[24] 120
Xaa[25] Trp Tyr Phe Asp Val Glu Arg Asn Ser 130
Cys Asn Asn Phe Xaa[26] Tyr Xaa[27] Gly Cys Xaa[28] 140
Xaa[29] Xaa[30] Lys Asn Ser Tyr Xaa[31] Ser Glu Glu 150
Ala Cys Met Leu Arg Cys Phe Arg Xaa[32] Gln 160
Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys 170
Val Val Val Leu Ala Gly Ala Val Ser 179
(SEQ ID NO: 11).

where Xaa[1] - Xaa[32] each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa[1]-Xaa[32] is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa^1 is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa^1 is His or Pro; or wherein Xaa^2 is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa^2 is Val or Thr; or wherein Xaa^3 is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa^3 is Arg or Pro; or wherein Xaa^4 is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa^4 is Arg or Lys; or wherein Xaa^5 is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa^5 is Ala; or wherein Xaa^6 is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa^6 is Ser or Arg ; or wherein Xaa^7 is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^7 is Met or Ile; or wherein Xaa^8 is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa^8 is Pro or Ile; or wherein Xaa^9 is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^9 is Arg: or wherein Xaa^{10} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa^{10} is Val; or wherein Xaa^{11} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{11} is Gly; or wherein Xaa^{12} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa^{12} is Asp or Arg; or wherein Xaa^{13} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{14} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{15} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{15} is Leu or Lys; or wherein Xaa^{16} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa^{16} is Val or Ala; or wherein Xaa^{17} is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa^{17} is Glu or Pro; or wherein Xaa^{18} is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa^{18} is Thr; or wherein Xaa^{19} is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa^{19} is Pro; or wherein Xaa^{20} is an amino acid residue selected from the group consisting of Arg, Lys, Gln and Ser, in

particular wherein Xaa^{20} is Arg or Lys; or wherein Xaa^{21} is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa^{21} is Ala; or wherein Xaa^{22} is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa^{22} is Ser or Arg ; or wherein Xaa^{23} is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^{23} is Phe or Ile; or wherein Xaa^{24} is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa^{24} is Pro or Ile; or wherein Xaa^{25} is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^{25} is Arg: or wherein Xaa^{26} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa^{26} is Val or Ile; or wherein Xaa^{27} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{27} is Gly; or wherein Xaa^{28} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa^{28} is Arg; or wherein Xaa^{29} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{30} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{31} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{31} is Arg or Lys; or wherein Xaa^{32} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa^{32} is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14), and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an

open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migrati n was from top to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular

size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with $CaCl_2$. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient und rgoing surgery, or with major trauma.

The present invention also provides methods for reducing perioperative

blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects, as well as the cost of surgery. The methods are thus useful in reducing blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery, as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40 µg/ml (see Pixley, et al. (1993) *Meth. in Enz.*, 222, 51-64) and is activated by tissue and plasma kallikrein. Once activated, it participates in the intrinsic pathway of blood coagulation which is activated when blood or plasma contacts a "foreign" or ani nic surface. Once activated, Factor XIIa can then

cleav and activate a number of other plasma proteases including Factor XI, prekallikrein, and C1 of the complement system. Thus Factor XII may be involved in causing hypotensive reactions since activated kallikrein can cleave kininogen releasing bradykinin (see Colman, (1984) *J. Clin. Invest.*, 73, 1249).

Sepsis is a disease that results from bacterial infection due to bacterial endotoxin or lipopolysaccharide (LPS). Exposure of Factor XII to LPS results in the activation of Factor XII. Patients with sepsis frequently have symptoms of intravascular coagulation which may also be due to activation of Factor XII by LPS. Septic shock can result from bacterial infection and is associated with fever, low systemic vascular resistance, and low arterial pressure. It is a common cause of death in intensive care units in the United States, where seventy five percent of the patients that die from septic shock have a persistent hypotension (see Parillo, et al. (1989) *Ann Rev. Med.*, 40, 469-485).

Adult respiratory distress syndrome is characterized by pulmonary edema, hypoxemia, and decreased pulmonary compliance. The pathogenesis of the disease is currently unknown although the proteolytic pathways of coagulation and fibrinolysis are believed to play a role (see Carvalho, et al. (1988) *J. Lab Clin. Med.*, 112: 270-277).

The proteins of the instant invention are also a novel human Kunitz type inhibitor of kallikrein, an activator of Factor XII. Thus another object of the current invention is to present a method for the prophylactic or therapeutic treatment of systemic inflammatory reactions such as septic shock, adult respiratory distress syndrome (ARDS), preeclampsia, multiple organ failure and disseminated intravascular coagulation (DIC). The therapeutic or prophylactic administration of the peptides of the instant invention would result in the modulation of these inflammatory conditions and be beneficial to the patient.

Plasmin plays an important role in extracellular matrix degradation and the activation of matrix-metallo protease (MMP) cascades. Collectively these proteases mediate migration of and tissue invasion by both endothelial cells during angiogenesis/neovascularization, and cancer cells during metastasis. Neovascularization is essential to support tumor growth and metastasis is a process which mediates the spreading of tumors and which is associated with extremely poor patient prognosis.

Several preclinical studies suggest that Kunitz like serine protease inhibitors with a protease specificity similar to aprotinin are useful as medicaments for cancer. For example, aprotinin reduced tumor growth and invasion, with increased tumor necrosis when administered to hamsters bearing a highly invasive fibrosarcoma or to mice bearing a similarly malignant mammary carcinoma (Latner et al., (1974), Br. J. Cancer 30: 60-67; Latner and Turner, (1976), Br. J. Cancer 33: 535-538). Furthermore, administration of 200,000 KIU of aprotinin i.p. to C57B1/6 Cr male mice on days 1 to 14 post-inoculation with Lewis lung carcinoma cells, reduced pulmonary metastases by 50% although had no effect on primary tumor mass (Giraldi et al., (1977) Eur. J. Cancer, 13: 1321-1323). Similarly, administration of 10,000 KIU i.p. on each of days 13-16 post-inoculation of C57BL/6J mice with Lewis tumor cells inhibited pulmonary metastases by 90% without affecting the primary tumor growth (Uetsuji et al., (1992), Jpn. J. Surg. 22: 429-442). In this same study, administration of plasmin or kallikrein with the same dosing schedule was argued to increase the number of pulmonary metastases. These results

prompted the authors to suggest that perioperative administration of aprotinin to cancer patients may reduce the likelihood of metastases. Black and Steger (1976, Eur. J. Pharmacol., 38: 313-319) found that aprotinin inhibited the growth of the transplanted rodent Murphy-Strum lymphosarcoma in rats and suggested that the effect involved the inhibition of the kinin-forming enzyme system. Twice daily i.p. injection of female ddY mice with 10,000 KIU of aprotinin for 7 weeks to mice each bearing a single autochtonous squamous cell carcinoma resulting from 3-methylcholanthrene treatment reduced the growth rate of the primary tumors by 90%. In some animals tumor regression was observed. While all vehicle treated animals had died within the seven weeks, all of the aprotinin treatment group remained alive. Reduced tumor growth was associated with hyperkeratosis (Ohkoshi, Gann (1980), 71: 246-250).

Clinically, a surgically cured group of 26 patients who received aprotinin i.v. exhibited a 70% survival two years post surgery with no recurrence of tumors whereas a placebo group of 26 patients at the same time exhibited only a 38% survival with a significant rate of tumor recurrence (Freeman et al. Br. Soc. Gastroenterol. (1980) supplement A: 902). In a case study (Guthrie et al., Br. J. Clin. Pract (1981) 35: 330-332), administration of bromocriptine plus aprotinin to a patient with advanced cancer of the cervix caused remission. Aprotinin was administerd both as a 500,000 KIU i.p. bolus every eight hours concurrently with a continuous i.v. infusion of aprotinin at a rate of 200,000 KIU per 6 hr for a total of seven days once a month. Treatment was ended at the end of the fourth month due to the development of an allergic reaction to aprotinin. More recent evidence has further underscored a role of plasmin as a target for these effects of aprotinin on metastases.

The mechanism for these events could be related to the fact that aprotinin blocks the invasive potential of cancer cell lines (Liu G., et al., Int J. Cancer (1995), 60: 501-506). Furthermore, since the proteins of the instant invention are also potent inhibitors of plasmin and kallikrien, they are contemplated for use as anti-cancer agents. For example they are contemplated for use in blocking primary tumor growth by restricting neovascularization, primary tumor invasion and in blocking metastasis through inhibition of tissue infiltration. The compounds may be administered locally to tumors r systemically. In a preferred mode f treatment, the protein would be administered perioperatively during tumor debulking to minimize the risk of metastasis. In such a regime, the blood sparing properties of the compound would be additionally advantageous in providing a clearer surgical field of

view. Another preferred mode of administration would be as a combination therapy with either MMP inhibitors or chemotherapy. An additional preferred mode of administration would be as a locally administered gene therapy designed to achieve selective expression of placental bikunin within the tumor cells, or their associated stroma and vascular beds.

Preferred types of cancers targeted for therapy would be vasular-dependent solid tumors such as breast, colon, lung, prostate and ovarian carcinomas which exhibit a high metastatic potential, and those for which local delivery of a high concentration of the protein is feasible such as lung cancers through pulmonary delivery, colon carcinomas through hepatic delivery to liver metastasis, or skin cancers such as head and neck carcinomas or melanomas through subcutaneous delivery. Since the proteins of the present invention are of human origin they would be less likely to be associated with allergic or anaphylactic reactions of the kind observed by Guthrie et al., *supra*, upon reuse.

Additionally, the proteins of the present invention are contemplated for use in the reduction of thromboembolic complications associated with activation of the intrinsic pathway of coagulation. This would include prevention of pulmonary embolism in late stage cancer patients, a frequent cause of death (Donati MB., (1994), Haemostasis 24: 128-131).

Edema of the brain and spinal cord is a complication resulting from traumatic brain or spinal cord injury, stroke, cerebral ischemia, cerebral and sub-arachnoid hemhorrhage, surgery (including open heart surgery), infectious diseases such as encephalitis and meningitis, granulomatous diseases such as Sarcoid and focal or diffuse carcinomas, and is a contributor to the high level of morbidity and death following these events. Bradykinin is known to disrupt the blood brain barrier experimentally (Greenwood J., (1991), Neuroradiology, 33: 95-100; Whittle et al., (1992), Acta Neurochir., 115: 53-59), and infusion of bradykinin into the internal carotid artery induced brain edema in spontaneously hypertensive rats (SHR) subjected to common carotid artery occlusion (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191). Elevated levels of bradykinin are found in extracellular fluids following trauma in a model involving traumatized rat spinal chord (Xu et al., (1991), J. Neurochem, 57: 975-980), and in plasma and tissue from rats with brain edema resulting from cerebral ischaemia (Kamiya et al., (1993), Stroke, 24: 571-575). Bradykinin is released from high molecular weight kininogen by serine proteases including kallikrein (Coleman (1984) J. Clin Invest., 73: 1249), and the serine protease inhibitor aprotinin was found to block the magnitude of brain edema resulting from

cerebralschemia in SHR rats (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191; Kamiya et al., (1993), Stroke, 24: 571-575) and rabbits subjected to a cold lesion of the brain (Unterberg et al., (1986), J. Neurosurgery, 64: 269-276).

These observations indicate that brain edema results from local proteolytic release of kinins such as bradykinin from high molecular weight kininogen, followed by bradykinin-induced increases in blood brain barrier permeability. Accordingly, placental bikunin and fragments thereof are contemplated as medicaments for the prevention of edema in patients at risk for this condition, particularly those of high risk of mortality or brain injury. This would include head and spinal trauma patients, polytrauma patients, patients undergoing surgery of the brain or spinal cord and their associated vessels or other generalsurgeries including open-heart surgery, patients who have suffered from a stroke, cerebral or sub-arachnoid hemorrhage, infectious diseases of the brain, granulomatous disease of the brain or diffuse or focal carcinomas and tumors of the brain or any conditions such as multiple sclerosis involving breakdown of the blood brain barrier or patients suffering from any other inflammatory processes of the brain or spinal cord. Patients would receive an administration of placental bikunin either as an infusion or bolus injection, intravenously or intracranially. Additional doses of placental bikunin could be administered intermittently over the following one to three weeks. Dose levels would be designed to attain circulating concentrations in excess of those required to neutralize elevations in plasma levels or bradykinin and other vasoactive peptides formed through the action of serine proteases, and sufficient to reduce edema. Since the protein is of human origin, repeated administration in this course of therapy would not lead to development of an immune reaction to the protein. Placental bikunin and fragments thereof would be contemplated for monotherapy or prophylacsis as well as for use in combination with other medicaments such as neurotherapeutics and neuroprotectants.

Recent evidence (Dela Cadena R. A. at al., (1995), FASEB J. 9: 446-452) has indicated that the contact activation pathway may contribute to the pathogenesis of arthritis and anemia, and that kallikrein inhibitors may be of therapeutic benefit. Accordingly, protease inhibitors of the present invention are contemplated according to their capacity to inhibit human kallikrein, as medicaments for the treatment of arthritis and anemia in humans.

Treatment of male non-insulin diabetic (NIDDM) patients with aprotinin significantly improved total glucose uptake and decreased the metabolic clearance rate of insulin (Laurenti et al., (1996), Diabetic Medicine 13: 642-645). Accordingly, the human proteins of the present invention are contemplated for chronic use as

medicaments for the treatment of NIDDM.

Daily treatment of patients at risk of preterm delivery with urinary trypsin inhibitor for two weeks significantly reduced recurrent uterine contractions (Kanayama et al., (1996), Eur J. Obstet. Gynecol. & Reprod. Biol. 67: 133-138). Accordingly, the human proteins of the present invention are contemplated for use in the prevention of preterm delivery.

Aprotinin has been shown to stimulate differentiation of mouse myoblasts in culture (Wells and Strickland, Development, (1994), 120: 3639-3647)), a process that is inhibited by TGFb. TGFb exists as an inactive pro-polypeptide which is activated by limited proteolysis. The mechanism of aprotinin action has been proposed to involve inhibition of proteases which process pro-TGFb to the mature active form. TGFb has been shown to be up-regulated in various fibrotic lesions and has long thought to be a potential target for anti-fibrotic therapies. In a rat model of pulmonary fibrosis for example, TGF-b concentrations paralleled the extent of bleomycin-induced inflammation. Furthermore, plasmin levels in the alveolar macrophage coincided with mature TGF-b levels, and the addition of the plasmin inhibitor a-2-antiplasmin abrogated the post translational activation of pro-TGFb by the macrophage (Khal et al., (1996), Am. J. Respir. Cell Mol. Biol. 15: 252-259.) The data suggest that plasmin contributes to the formation of active TGFb by alveolar macrophage, and that this process plays a pathologic role in the bleomycin-induced lung inflammation.

In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various fibrotic disorders, including pulmonary, hepatic, renal and dermal (scleroderma) fibrosis.

Aerosilized aprotinin was shown to protect >50% of mice infected with lethal doses of either influenza virus or paramyxovirus (Ovcharenko and Zhirnov, Antiviral Research, (1994), 23: 107-118). A suppression of the development of fatal hemorrhagic bronchopneumonia and a normalization of body weight gain were also noted with aerosilized aprotinin treatment. In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various respiratory related influenza-like diseases.

The human placental bikunin, isolated domains, and other variants of the invention are contemplated for use in the medical/therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitory profiles, in particular those which necessitate usage of large doses. These would include diseases f r which use of the human protein is indicated by virtue of its ability to inhibit human serine proteases such as trypsin, plasmin,

kallikrein, elastase, cathepsin G and proteinase-3, which include and are not limited to: acute pancreatitis (pancreatic elastase and trypsin), inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, various forms of shock, including shock lung, endotoxin shock and post operative complications; disturbances of blood coagulation such as hyperfibrinolytic hemorrhage; acute and chronic inflammatory reactions, in particular for the therapy and prophylaxis of organ lesions, such as for example pancreatitis and radiation induced enteritis, complex-mediated inflammatory reactions such as immunovasculitis, glomerulonephritis and types of arthritis; collagenoses in particular rheumatoid arthritis; types of arthritis caused by metabolism-related deposits (for example gout); degeneration of the elastic constituents of the connective tissue parts of organs, such as in atherosclerosis (serum elastase) or pulmonary emphysema (neutrophil elastase); adult respiratory distress syndrome, inflammatory bowel disease, and psoriasis.

A major unexpected finding was that the synthetic peptides encoding bikunin (7-64), and bikunin (102-159), could properly fold into the correct three-dimensional conformation having active protease inhibitor bioactivity (Examples 2 and 1, respectively). Upon folding, each of these fragments of Bikunin underwent a reduction in mass of 6 mass units, consistent with the formation in each case, of three intrachain disulfide bonds between six cysteine residues of each fragment. Another surprising finding is that the synthetic peptides encoding bikunin (7-64), bikunin (102-159), and bikunin (1-170) are highly inhibitory of plasmin and both tissue and plasma kallikrein (Example 4 , 3, and 10 respectively). Inhibition of plasmin and kallikrein by Trasylol® is thought to be involved in the mechanism by which Trasylol® reduces blood loss during open heart surgery. Our unexpected findings of the specificity of the Kunitz domains of the present invention make them suitable therapeutic agents for blood sparing during surgery or trauma where there is significant blood loss, or for any other condition where inhibition of plasmin and/or kallikrein would be beneficial.

Furthermore, we showed in this disclosure (Example 10) that placental bikunin (1-170) is a potent inhibitor of factor XIa and a moderate inhibitor of factor Xa. Factor XIa plays an essential role in the intrinsic pathway of coagulation, serving to interconvert inactive factor IX into active factor IXa. Thus, Placental Bikunin inhibits two key enzymes of the intrinsic pathway, kallikrein and factor XIa. Consistent with these observations, we also showed that placental bikunin (1-170) is a potent inhibitor of the activated partial

thromboplastin time, which is a measure of th speed of coagulation driven by the intrinsic pathway. On the other hand, we showed that Placental bikunin (1-170) is an extremely weak inhibitor of the tissue factor VIIa complex, suggesting that it is not important in the regulation of the extrinsic coagulation cascade. Based on these unexpected findings, placental bikunin is contemplated as a medicament for diseases in which activation of the intrinsic pathway of coagulation contributes significantly to the disease mechanism. Examples of such diseases would include post-traumatic shock and disseminated intravascular coagulation.

A significant advantage of the Kunitz domains of the present invention is that they are human proteins, and also less positively charged than Trasylol® (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly reduced risk of undesired immunological reactions as compared to administration of similar doses of Trasylol®. Furthermore, it was found that bikunin (102-159), bikunin (7-64), and bikunin (1-170) are significantly more potent inhibitors of plasma kallikrein than Trasylol® *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* at lowering blood loss in patients.

The amount of serine protease inhibitor administered should be sufficient to provide a supra normal plasma level. For the prophylactic reduction of bleeding during and following coronary aortic by-pass surgery (CABG), the proteins of the instant invention may be used in place of Trasylol® while taking into account the differences in potency. The use of Trasylol® is outlined in the Physicians Desk Reference, (1995), listing for Trasylol® supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given slowly over about 20 to 30 minutes, after induction of anesthesia but prior to sternotomy. In general, a total dose of between about $2x10^6$ KIU (kallikrein inhibitory units) and 8 $X10^6$ KIU will be used, depending on such factors as patient weight and the length of the surgery. Preferred loading doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU). When the loading dose is complete, it is followed by the constant infusion dose, which is continued until surgery is complete and the patient leaves the perating room. Preferred constant infusion doses are in the range of about 250,000 to 500,000 KIU per hour. The pump prime dose is added to the priming fluid of the

cardiopulmonary bypass circuit, by replacement of an aliquot of the priming fluid prior to the institution of the cardiopulmonary bypass. Preferred pump prime doses are those that contain a total of about one to two million KIU.

The proteins of the instant invention are employed in pharmaceutical compositions formulated in the manner known to the art. Such compositions contain active ingredient(s) plus one or more pharmaceutically acceptable carriers, diluents, fillers, binders, and other excipients, depending on the administration mode and dosage form contemplated. Examples of therapeutically inert inorganic or organic carriers known to those skilled in the art include, but are not limited to, lactose, corn starch or derivatives thereof, talc, vegetable oils, waxes, fats, polyols such as polyethylene glycol, water, saccharose, alcohols, glycerin and the like. Various preservatives, emulsifiers, dispersants, flavorants, wetting agents, antioxidants, sweeteners, colorants, stabilizers, salts, buffers and the like can also be added, as required to assist in the stabilization of the formulation or to assist in increasing bioavailability of the active ingredient(s) or to yield a formulation of acceptable flavor or odor in the case of oral dosing. The inhibitor employed in such compositions may be in the form of the original compound itself, or optionally, in the form of a pharmaceutically acceptable salt. The proteins of the instant invention can be adminstered alone, or in various combinations, and in combination with other therapeutic compositions. The compositions so formulated are selected as needed for administration of the inhibitor by any suitable mode known to those skilled in the art.

Parenteral administration modes include intravenous (*i.v.*), subcutaneous (*s.c.*), intraperitoneal (*i.p.*), and intramuscular (*i.m.*) routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed. Alternatively, the drug can be administered at a desired rate continuously by *i.v.* catheter. Suitable vehicles include sterile, non-pyrogenic aqueous diluents, such as sterile water for injection, sterile-buffered solutions or sterile saline. The resulting composition is administered to the patient prior to and/or during surgery by intravenous injection or infusion.

Improved half-life and targeting of the drug to phagosomes such as neutrophils and macrophage involved in inflammation may be aided by entrapment of the drug in liposomes. It should be possible to improve the selectivity of liposomal targeting by incorporating into the outside of the liposomes ligands that bind to macromolecules specific to target organs/tissues such as the GI tract and lungs. Alternatively, *i.m.* or *s.c.* deposit injection with

or without encapsulation of the drug into degradable microspheres (e.g., comprising poly-DL-lactide-co-glycolide) or protective formulations containing collagen can be used to obtain prolonged sustained drug release. For improved convenience of the dosage form it is possible to use an *i.p.* implanted reservoir and septum such as the percuseal system. Improved convenience and patient compliance may also be achieved by use of either injector pens (e.g., the Novo Pin or Q-pen) or needle-free jet injectors (e.g., from Bioject, Mediject or Becton Dickinson). Precisely controlled release can also be achieved using implantable pumps with delivery to the desired site via a cannula. Examples include the subcutaneously implanted osmotic pumps available from ALZA such as the ALZET osmotic pump.

Nasal delivery may be achieved by incorporating the drug into bioadhesive particulate carriers (<200 mm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption enhancers such as phospholipids or acylcarnitines. Commercially available systems include those developed by Dan Biosys and Scios Nova.

Pulmonary delivery represents a nonparenteral mode of administration of the drug to the circulation. The lower airway epithelia are highly permeable to a wide range of proteins of molecular sizes up to about 20 kDa. Micron-sized dry powders containing the medicament in a suitable carrier such as mannitol, sucrose or lactose may be delivered to the distal alveolar surface using dry powder inhalers such as those of Inhale™, Dura™, Fisons (Spinhaler™), and Glaxo (Rotahaler™), or Astra (Turbohaler™) propellant based metered dose inhalers. Solution formulations with or without liposomes may be delivered using ultrasonic nebulizers.

Oral delivery may be achieved by incorporating the drug into tablets, coated tablets, dragées, hard and soft gelatin capsules, solutions, emulsions, suspensions or enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples of the latter include the OROS-CT/Osmet™ system of ALZA, and the PULSINCAP™ system of Scherer Drug Delivery Systems. Other systems use azo-crosslinked polymers that are degraded by colon-specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH in the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers. Rectal delivery may be achieved by incorporating th drug into suppositories.

In its preferred medicinal application, for reduction of perioperativ

blood loss, the preferred mode of administration of the placental bikunin variants of the present invention is parenterally, preferably by *i.v.* route through a central line.

The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, based on a determination of the amount of target protease such as plasmin or kallikrein which must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent.

Additionally, placental bikunin, isolated domains or other variants may be used to isolate natural substances such as its cognate proteases from human material using affinity based separation methods, as well as to elicit antibodies to the protease that can be further used to explore the tissue distribution and useful functions of Placental bikunin.

Searching Human Sequence Data

The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI (National Center for Biological Information, Maryland). Using the TBlastN algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215: 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, Trasylol®. This search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library. The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the

longest translated open reading frame from clone R74593 (SEQ ID NO: 15) contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been sequenced incorrectly.

Discovery of Human Bikunin

To specifically isolate and determine the actual human sequence, cDNA primers were designed to be capable of hybridizing to sequences located 5' and 3' to the segment of cDNA encoding our proposed Kunitz like sequences found within R35464 and R74593. The primers used to amplify a fragment encoding the Kunitz like sequence of R74593 were CGAAGCTTCATCTCCGAAGCTCCAGACG (the 3'primer with a HindIII site; SEQ ID NO:33) and AGGATCTAGACAATAATTACCTGACCAAGGA (the 5'primer with an XbaI site; SEQ ID NO:34).

These primers were used to amplify by PCR (30 cycles) a 500 base pair product from a human placental cDNA library from Clontech (MATCHMAKER, Cat #HL4003AB, Clontech Laboratories, Palo Alto, CA), which was subcloned into Bluescript-SK+ and sequenced with the T3 primer with a Sequenase™ kit version 2.0. Surprisingly, the sequence of the fragment obtained using our primers was different from the sequence listed in the dbEST data base for clone R74593. In particular, our new sequence contained an additional guanosine base inserted 3' to the putative stop codon, but 5' to the segment encoding the Kunitz-like sequence (Figure 3). The insertion of an additional G shifted the stop codon out of the reading frame for the Kunitz-like domain (G at base pair 114 of the corrected sequence for R74593; Figure 3).

Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it contained all six of the characteristic cysteines. Overlay of each of the nucleotide sequences with that of R74593 (corrected by the insertion of G at b,p, 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3;

full translation SEQ ID NO: 10) that contained two complete Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

Further efforts attempted to obtain additional 5' sequence by querying dbEST with the sequence of R35464. Possible matches from such searches, that possessed additional 5' sequence were then in turn used to re-query the dbEST. In such an iterative fashion, a series of overlapping 5' sequences were identified which included clones H16866, T66058, R34808, R87894, N40851 and N39876 (Figure 4). Alignment of some of these sequences suggested the presence of a 5' ATG which might serve as a start site for synthesis of the consensus translated protein sequence. From this selected information, it was now possible to selectively screen for, and determine the nucleic acid and polypeptide sequences of a human protein with homologous function to aprotinin.

Re-interrogation of the dbEST revealed a number of new EST entries shown schematically in Figure 4B. Overlap with these additional ESTs allowed us to construct a much longer consensus oligonucleotide sequence (Figure 4C) that extended both 5' and 3' beyond the original oligonucleotide sequence depicted in Figure 3. In fact, the new sequence of total length 1.6 kilobases extended all the way to the 3' poly-A tail. The increased number of overlapping ESTs at each base-pair position along the sequence improved the level of confidence in certain regions such as the sequence overlapping with the 3' end of EST R74593 (Figure 3). Several overlapping ESTs in this region corroborated two critical base deletions relative to R74593 (located as bold underlined in Figure 4C, map positions 994 and 1005). Translation of the new consensus sequence (Figure 4D) in the bikunin encoding frame yielded a form of placental bikunin that was larger (248 amino acids) than the mature sequence (179 amino acids) encoded from the original consensus (SEQ ID NO: 1), and was terminated by an in-frame stop codon within the oligonucleotide consensus. The size increase was due to a frame shift in the 3' coding region resulting from removal of the two base insertions unique to EST R74593. The frame shift moved the stop codon of the original consensus (Figure 3) out of frame enabling read through into a new frame encoding the additional amino acid sequence. The new translation product (Figure 4D) was identical to the original protein consensus sequence (SEQ ID NO: 1) between residues +1 to +175 (encoding the Kunitz domains), but contained a new C-terminal extension exhibiting a putative 24 residue long transmembrane domain (underlined in Figure 4D) followed by a short 31 residue cytoplasmic domain. The precise sequence

around the initiator methionine and signal peptide was somewhat tentative due to considerable heterogeneity amongst the overlapping ESTs in this region.

Analysis of the protein sequence by Geneworks™, highlighted asparagine residues at positions 30 and 67 as consensus sites for putative N-linked glycosylation. Asparagine 30 was not observed during N-terminal sequencing of the full length protein isolated from human placenta, consistent with it being glycosylated.

Cloning of Human Bikunin

The existence of a human mRNA corresponding to the putative human bikunin nucleotide sequence inferred from the analysis of Figure 3, was confirmed as follows. The nucleic acid primer hybridizing 5' to the Kunitz-encoding cDNA sequence of R35464 (b.p. 3-27 of consensus nucleotide sequence in Figure 3):

GGTCTAGAGGCCGGGTCGTTTCTCGCCTGGCTGGGA

(a 5' primer derived from R35464 sequence with an XbaI site; SEQ ID NO: 35), and the nucleic acid primer hybridizing 3' to the Kunitz encoding sequence of R74593 (b.p. 680-700 of consensus nucleotide sequence in Figure 3), was used to PCR amplify, from a Clontech human placental library, a fragment of the size (ca. 670 b.p) expected from a cDNA consensus nucleotide sequence encoding the placental bikunin sequence of Figure 3 (Shown schematically in Figure 4A).

Using a 5' primer hybridizing to a sequence in R87894 that is 126 b.p 5' to the putative ATG start site discussed above, (shown schematically in Figure 4A at b.p. 110) plus the same 3' primer to R74593 as used above, it was possible to amplify a fragment from a Clontech human placental library of the expected size (approximately 872 b.p) predicted by EST overlay (Shown schematically in Figure 4).

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5' end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3' end. This 3' nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159).

To obtain a cDNA encoding the entire extracellular region of the protein, the following 5' PCR primer:

CACCTGATCGCGAGACCCC (SEQ ID NO: 36)

designed to hybridize to a sequence within EST R34808 was used with the same 3' primer to EST 74593 to amplify (30 cycles) an approximately 780 base-pair

cDNA product from the human placental cDNA library. This product was gel purified, and cloned into the TA vector (Invitrogen) for DNA sequencing by the dideoxy method (Sanger F., et al., (1977) Proc. Natl. Acad. Sci (USA), 74: 5463-5467) with the following primers:

Vector Specific:	GATTTAGGTGACACTATAG (SP6)	(SEQ ID NO: 37)
	TAATACGACTCACTATAGGG (T7)	(SEQ ID NO: 38)
Gene Specific:	TTACCTGACCAAGGAGGAGTGC	(SEQ ID NO: 39)
	AATCCGCTGCATTCCTGCTGGTG	(SEQ ID NO: 40)
	CAGTCACTGGGCCTTGCCGT	(SEQ ID NO: 41)

The resulting cDNA sequence is depicted in Figure 4E together with its translation product. At the nucleotide level, the sequence exhibited only minor differences from the consensus EST sequence (Figure 4D). Translation of the sequence yielded a coding sequence containing an in-frame initiator ATG site, signal peptide and mature placental bikunin sequence and transmembrane domain. The translated sequence of the PCR product was missing the last 12 amino acid residues from the cytoplasmic domain as a consequence of the choice of selection of the 3' primer for PCR amplification. This choice of 3' PCR primer (designed based on the sequence of R74593) was also responsible for the introduction of an artifactual S to F mutation at amino acid position 211 of the translated PCR-derived sequence. The signal peptide deduced from translation of the PCR fragment was somewhat different to that of the EST consensus.

To obtain a full length placental bikunin cDNA, the PCR derived product (Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with ^{32}P-CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, Unizap™ λ library) using colony hybridization techniques. Approximately 2 X 10^6 phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin

sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7). Both the natural protein and the form of the protein expressed in Sf9 cells are probably glycosylated at the asparagine residue at position 30 based on the recoveries of PTH-amino acids during N-terminal sequencing (Examples 7 and 9).

Based on the above observations, it seems that full length placental bikunin has the capacity to exist as a transmembrane protein on the surface of cells as well as a soluble protein. Other transmembrane proteins that contain Kunitz domains are known to undergo proteolytic processing to yield mixtures of soluble and membrane associated forms. These include two forms of the Amyloid Precursor Protein termed APP751 (Esch F., et al., (1990) Science, 248: 1122-1124) and APP 770 (Wang R., et al., (1991), J. Biol Chem, 266: 16960-16964).

Contact activation is a process which is activated by exposure of damaged vascular surfaces to components of the coagulation cascade. Angiogenesis is a process that involves local activation of plasmin at endothelial surfaces. The specificity of placental bikunin and its putative capacity to anchor to cell surfaces, suggest that the physiologic functions of transmembranous placental bikunin may include regulation of contact activation and angiogenesis.

The amino acid sequences for placental bikunin (7-64), bikunin (102-159), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFastA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85: 2444-2448), these same protein sequences were searched versus the six-frame translations f the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL

(modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

Isolation of Human Bikunin

As mentioned above, synthetic peptides corresponding to bikunin (7-64) and bikunin (102-159) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate native placental bikunin from human tissue.

Using a purification scheme which employed kallikrein-sepharose affinity chromatography as a first step, highly purified native potent kallikrein inhibitor was isolated. The isolated native human bikunin had an identical N-terminus (sequenced for 50 amino acid residues) as the sequence predicted by the translation of the consensus nucleic acid sequence (Figure 3) amino acid residues +1 to +50 (Example 7). This confirmed for the first time the existence of a novel native kallikrein inhibitor isolated from human placenta.

Known Kunitz-like domains are listed below. Residues believed to be making contact with target proteases are highlighted as of special interest (bold/underlined). These particular residues are named positions Xaa^{1-16} for specific reference as shown by label Xaa below:

```
Xaa                                  1 1  111     1       1
 1        2  3 456789                0 1  234     5       6
 1) IHDFCLVSKVV GRCRASMPRW WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATV
 2) YEEYCTANAVT GPCRASFPRW YFDVERNSCN NFIYGGCRGN KNSYRSEEAC MLRCFRQ
 3) -HSFCAFKADD GPCKAIMKRF FFNIFTRQCE EFIYGGCEGN QNRFESLEEC KKMCTRD
 4) -PDFCFLEEDP GICRGYITRY FYNNQTKQCE RFKYGGCLGN MNNFETLEEC KNICEDG
 5) -PSWCLTPADR GLCRANENRF YYNSVIGKCR PFKYSGCGGN ENNFTSKQEC LRACKKG
 6) -AEICLLPLDY GPCRALLLRY YYRYRTQSCR QFLYGGCEGN ANNFYTWEAC DDACWRI
 7) -PSFCYSPKDE GLCSANVTRY YFNPRYRTCD AFTYTGCGGN DNNFVSREDC KRACAKA
 8) -KAVCSQEAMT GPCRAVMPRT TFDLSKGKCV RFITGGCGGN RNNFESEDYC MAVCKAM
 9) RPDFCLEPPYT GPCKARIIRY FYNAKAGLCQ TFVYGGCRAK RNNFKSAEDC MRTCGGA
10) ----CQLGYSA GPCMGMTSRY FYNGTSMACE TFQYGGCMGN GNNFVTEKEC LQTC
11)  VAACNLPIVR GPCRAFIQLW AFDAVKGKCV LFPYGGCQGN GNKFYSEKEC REYCGVP
12) -EVCCSEQAET GPCRAMISRW YFDVTEGKCA PFFYGGCGGN RNNFDTEEYC MAVCGSA
13) ----CKLPKDE GTCRDFILKW YYDPNTKSCA RFWYGGCGGN ENKFGSQKEC EKVC
14) -PNVCAFPMEK GPCQTYMTRW FFNFETGECE LFAYGGCGGN SNNFLRKEKC EKFCKFT
```

Where sequence number 1) is Bikunin (7-64) (SEQ ID NO: 4); sequence 2) is Bikunin (102-159) (SEQ ID NO: 6); sequence 3) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 18); sequence 4) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 19); sequence 5) is Tissue factor pathway inhibitor precursor (SEQ ID NO: 20); sequence 6) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 21); sequence 7) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 22); sequence 8) is Amyloid precursor protein homologue (SEQ ID NO: 23); sequence 9) is Aprotinin (SEQ ID NO: 24); sequence 10) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 25); sequence 11) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 26); sequence 12) is Amyloid precursor protein (SEQ ID NO: 27); sequence 13) is Collagen α-3(VI) precursor (SEQ ID NO: 28); and squence 14) is HKI-B9 (SEQ ID NO: 29).

It can be seen that Placental Bikunin (7-64) and (102-159) each have the same number (six) and spacing of cysteine residues as is found in members of the Kunitz class of serine protease inhibitors. The precise bonding of cysteine residues to form the three intrachain disulfide bonds is known and invarient for all previously known Kunitz family members (Laskowski, M et al., 1980, Ann. Rev. Biochem. 49:593-626). Based on this known bonding pattern and the fact that the folding of Placental Bikunin (7-64) and (102-159) into active protease inhibitors is accompanied by a mass reduction consistent with the formation of

three intrachain disulfide bonds (Examples 2 and 1), it is highly probable that the disulfide bonding within the Kunitz domains of Placental Bikunin occur between cysteine residues: C11 and C61; C20 and C44; C36 and C57; C106 and C156; C115 and C139; C131 and C152. Furthermore, this pattern of disulfide bonding is highly probable in larger forms of Placental Bikunin containing both Kunitz domains since such forms of the protein are also active serine protease inhibitors and because N-terminal sequencing (Example 7) of native Placental Bikunin for 50 cycles yielded a sequence that was silent at positions where th cysteine residues were expected.

The placental bikunin, isolated domains or other variants of the present invention may be produced by standard solid phase peptide synthesis using either t-Boc chemistry as described by Merrifield R.B. and Barany G., in: The peptides, Analysis, Synthesis, Biology, 2, Gross E. et al., Eds. Academic Press (1980) Chapter 1; or using F-moc chemistry as described by Carpino L.A., and Han G.Y., (1970) J. Amer Chem Soc., 92, 5748-5749, and illustrated in Example 2. Alternatively, expression of a DNA encoding the placental bikunin variant may be used to produce recombinant placental bikunin variants.

The invention also relates to DNA constructs that encode the Placental bikunin protein variants of the present invention. These constructs may be prepared by synthetic methods such as those described in Beaucage S.L. and Caruthers M.H., (1981) Tetrahedron Lett, 22, pp1859-1862; Matteucci M.D and Caruthers M.H., (1981), J. Am. Chem. Soc. 103, p 3185; or from genomic or cDNA which may have been obtained by screening genomic or cDNA libraries with cDNA probes designed to hybridize with placental bikunin encoding DNA sequence. Genomic or cDNA sequence can be modified at one or more sites to obtain cDNA encoding any of the amino acid substitutions or deletions described in this disclosure.

The instant invention also relates to expression vectors containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. The cDNA should be connected to a suitable promoter sequence which shows transcriptional activity in the host cell of choice, possess a suitable terminator and a poly-adenylation signal. The cDNA encoding the placental bikunin variant can be fused to a 5' signal peptide that will result in the protein encoded by the cDNA to undergo secreti n. The signal peptide can be one that is recognized by the host organism. In the case of a mammalian host cell, the signal peptide can also be the natural signal peptide

present in full length placental bikunin. The procedures used to prepare such vectors for expression of placental bikunin variants are well known in the art and are for example described in Sambrook et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, New York, (1989).

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as E. coli, and yeasts such as Saccharomyces cervisiae. Methods for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., Current Protocols in Molecular Biology, John Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) and (102-159), yeast and E. coli expression systems are preferable, with yeast systems being most preferred. Typically, yeast expression would be carried out as described in US patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159). E.coli expression could be carried out using the methods described in US patent 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin (7-159).

DNA encoding variants of placental bikunin that possess amino acid substitution of the natural amino sequence can be prepared for expression of recombinant protein using the methods of Kunkel T.A., (1985) Proc. Natl. Acad. Sci USA 82: 488-492. Briefly, the DNA to be mutagenized is cloned into a single stranded bacteriophage vector such as M13. An oligonucleotide spanning the region to be changed and encoding the substitution is hybridized to the single stranded DNA and made double stranded by standard molecular biology techniques. This DNA is then transformed into an appropriate bacterial host and verified by dideoxynucleotide sequ ncing. The correct DNA is then cloned into the expression plasmid. Alternatively, the target DNA may be mutagenized by standard PCR techniques, sequenced, and inserted into the appropriate expression plasmid.

The following particular examples are offered by way of illustration, and not limitation, of certain aspects and preferred embodiments of the instant invention.

Example 1

Preparation of synthetic placental bikunin (102-159)

Materials and methods/Reagents used. The fluorogenic substrate Tos-Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC, bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

Recombinant aprotinin (Trasylol®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich (Milwaukee, WI).

Quantification of functional placental bikunin (7-64) and (102-159)

The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37%C with bikunin (7-64) or (102-159), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM $CaCl_2$ and 0.01% triton X-100). GPK-AMC was added (20 µM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition for each was calculated according to equation 1; where R_O is the rate of fluorescence increase in the presence of inhibitor and R_1 is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.

$$\% \text{ inhibition} = 100 \times [1 - R_O/R_1] \qquad (1)$$

Synthesis. Placental bikunin (102-159) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of amino acid for each coupling. Cleavage and deprotection was

performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H_2O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy (MH+ =6836.1; calcd = 6835.5) for the predicted sequence:

YEEYCTANAV TGPCRASFPR WYFDVERNSC NNFIYGGCRG NKNSYRSEEA CMLRCFRQ (SEQ ID NO: 6)

Purification. Refolding of placental bikunin (102-159) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113: 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0 to obtain a final concentration of 0.5 mg/ml peptide in 20% DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active fractions were pooled (see below) and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitril in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized, redissolved in 0.1% TFA, and stored at -20°C until needed.

Results. Synthetic placental bikunin (102-159) was ref lded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

Table 1

Purification table for the isolation of synthetic placental bikunin (102-159)

TABLE 1

Purification Step	Vol (ml)	mg/ml	mg	Units[c] (U)	SpA (U/mg)	Yield
8.0 M Urea	4.0	3.75[a]	15.0	0	0	-
20% DMSO	32.0	0.47[a]	15.0	16,162	1,078	100
Kallikrein affinity	9.8	0.009[b]	0.09	15,700	170,000	97
C18	3.0	0.013[ab]	0.04	11,964	300,000	74

[a]Protein determined by AAA.
[b]Protein determined by OD280 nm using the extinction coefficient determined for the purified protein (1.7×10^4 $Lmol^{-1}$ cm^{-1}).
[c]One Unit is defined as the amount of material required to inhibit 50% of trypsin activity in a standard assay.

Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast Ampholine® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) was determined to be 8.3, in agreement with the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 152: 33-50).

Example 2

Preparation f synthetic placental bikunin (7-64)

Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

Results. The final purified reduced peptide exhibited an MH+ = 6563, consistent with the sequence:

IHDFCLVSKV VGRCRASMPR WWYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was active as an inhibitor of trypsin (Table 2 below).

Table 2A

Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2A

Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	8.0	2.5	20.0	0	0	-
20% DMSO	64.0	0.31	20.0	68,699	3,435	100
Kall affinity pH 4.0	11.7	0.10	1.16	43,333	36,110	62
Kall affinity pH 1.7	9.0	0.64	5.8	4972	857	7.2
C18-1	4.6	0.14	0.06	21,905	350,143	31.9
C18-2	1.0	0.08	0.02	7,937	466,882	11.5

The purified refolded protein exhibited an MH+ = 6558, i.e. 5±1 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of at least one appropriate disulfide bond.

The pI of placental bikunin (7-64) was determined using the methods employed to determine the pI f placental bikunin (102-159). Placental bikunin (7-64) exhibited a pI that was much higher than the predicted value (pI = 7.9).

Refolded placental bikunin (7-64) migrated to the cathodic edge of the gel (pH 9.5) and an accurate pI could not be determined under these conditions.

Continued Preparation of synthetic placental bikunin (7-64)

Because the synthetic placental bikunin (7-64) may not have undergone complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide (0.27 mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25 C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

Results. The final purified reduced peptide exhibited an MH+ = 6567.5, consistent with the sequence:

IHDFCLVSKV VGRCRASMPRW WYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was as active as an inhibitor of trypsin (Table 2B below).

Table 2B
Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2B

Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	4.9	2.1	10.5	0	0	-
20% DMSO	39.0	0.27	10.5	236,000	22,500	100
Kallikrein Affinity (pH 2)	14.5	0.3	0.43	120,000	279,070	50.9
C18 Reverse-Phase	0.2	1.2	0.24	70,676	294,483	30.0

The purified refolded protein exhibited an MH+ = 6561.2, i.e. 6.3 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of the expected three disulfide bonds.

The pI of refolded placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Refolded placental bikunin (7-64) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

Example 3

In vitro specificity of functional placental bikunin fragment (102-159)

Proteases. Bovine trypsin, human plasmin, and bovine pancreatic kallikrein quantitation was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase,T., and Shaw, E., (1970) Methods Enzmol., 19: 20-27). Human kallikrein was quantitated by active site titration using bovine aprotinin as a standard and PFR-AMC as a substrate assuming a 1:1 complex formation. The K_m for GPK-AMC with trypsin and plasmin under the conditions used for each enzyme was 29 µM and 726 µM, respectively; the K_m for PFR-AMC with human plasma kallikrein and bovine pancreatic kallikrein was 457 µM and 81.5 µM, respectively; the K_m for AAPR-AMC with elastase was 1600 µM. Human tissue kallikrein (Bayer, Germany) quantification was carried out by active site titration using p'nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) Methods Enzmol. 19: 20-27).

Inhibition Kinetics: The inhibition of trypsin by placental bikunin (102-159) or aprotinin was measured by the incubation of 50 pM trypsin with placental bikunin (102-159) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15 µl of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25 µl of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at 37°C 15 µl of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100 µM. The apparent inhibition constant K_i^* was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): Th kinetic data from

each experiment were analyzed in terms of the equation for a tight binding inhibitor:

$$V_i/V_o = 1 - (E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4 E_o I_o]^{1/2})/2E_o \quad (2)$$

where V_i/V_o is the fractional enzyme activity (inhibited vs. uninhibited rate), and E_o and I_o are the total concentrations of enzyme and inhibitor, respectively. Ki values were obtained by correcting for the effect of substrate according to the equation:

$$K_i = K_i^*/(1 + [S_o]/K_m) \quad (3)$$

(Boudier, C., and Bieth, J. G., (1989) Biochim Biophys Acta., 995: 36-41)

For the inhibition of human neutrophil elastase by placental bikunin (102-159) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (150 nM) or aprotinin (0-7.5 μM) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37%C, AAPM-AMC (500 μM or 1000 μM) was added and the fluorescence measured over a two-minute period. Ki values were determined from Dixon plots of the form 1/V versus [I] performed at two different substrate concentrations (Dixon et al., 1979).

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) or placental bikunin fragment (102-159) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (0-40 nM) or placental bikunin (102-159) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10 uM final and the change in fluorescence monitored. The Km for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7 uM. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at

37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64), 0 to 140 nM placental bikunin (102-159) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl2, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

Results: A direct comparison of the inhibition profiles of placental bikunin (102-159) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K_i values are listed in Table 3 below.

Table 3
Ki values for the inhibition of various proteases by bikunin (102-159)

TABLE 3

Protease (concentration)	bikunin (102-159) Ki (nM)	Aprotinin Ki (nM)	Substrate (concentration)	Km (mM)
Trypsin (48.5 pM)	0.4	0.8	GPK-AMC (0.03 mM)	0.022
Chymotrypsin (5 nM)	0.24	0.86	AAPF-pNA (0.08 mM)	0.027
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	PFR-AMC (0.1 mM)	0.08
Human Plasma Kallikrein (2.5 nM)	0.3	19.0	PFR-AMC (0.3 mM)	0.46
Human Plasmin (50 pM)	1.8	1.3	GPK-AMC (0.5 mM)	0.73
Human Neutrophil Elastase (19 nM)	323.0	8500.0	AAPM-AMC (1.0 µM)	1.6
Factor XIIa	>300.0	12,000.0	PFR-AMC (0.2 µM)	0.35
Human Tissue Kallikrein (0.35 nM)	0.13	0.004	PFR-AMC (10 µM)	0.0057
factor Xa (0.87 nM)	274	N.I. at 3 µM	LGR-AMC (0.6 mM)	N.D.
urokinase	11000	4500	GGR-AMC (0.7 mM)	N.D.
factor XIa (0.1 nM)	15	288	E(OBz)AR-AMC (0.4 mM)	0.46

Placental bikunin (102-159) and aprotinin inhibit bovine trypsin and human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a Ki of 8.5 µM. Placental bikunin (102-159) inhibited elastase with a Ki of 323nM. The K_i value for the placental bikunin (102-159) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) is a more potent

inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

Because placental bikunin (102-159) is greater than 50 times more potent than Trasylol® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159)) are needed than Trasylol® in order to maintain the effective patient doses of inhibitor in KIU. This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients. Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

Example 4

In vitro specificity of functional placental bikunin fragment (7-64)

In vitro specificity of functional human placental bikunin (7-64) was determined using the materials and methods as described in the Examples above.

Results: The table below shows the efficacy of placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4 A
Ki values for the inhibition of various proteases by bikunin(7-64)

TABLE 4A

Protease (concentration)	bikunin(7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (48.5 pM)	0.17	0.8	0.4
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	0.4
Human Plasma Kallikrein (2.5 nM)	2.4	19.0	0.3
Human Plasmin (50 pM)	3.1	1.3	1.8
Bovine chymotrypsin (5 nM)	0.6	0.9	0.2
Factor XIIa	>300	12000	>300
elastase	>100	8500	323

The results show that the amino acid sequence encoding plac ntal bikunin (7-64) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

Table 4B below also shows the efficacy of refolded placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4B
Ki values for the inhibition of various proteases by refolded bikunin (7-64)

TABLE 4B

Protease (concentration)	bikunin (7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (50 pM)	0.2	0.8	0.3
Human Plasma Kallikrein (0.2 nM)	0.7	19.0	0.7
Human Plasmin (50 pM)	3.7	1.3	1.8
Factor XIIa	not done	12,000	4,500
Factor XIa (0.1 nM)	200	288	15
Human Tissue Kallikrein	2.3	0.004	0.13

Suprisingly, placental bikunin (7-64) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

Example 5

Expression of placental bikunin variant (102-159) in yeast

The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO: 6) was generated using synthetic oligonucleotides. The final DNA product consisted (5' to 3') of 15 nucleotides from the yeast α-mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast α-mating factor propeptide fused to the 58 amino acid sequence of placental bikunin (102-159). Processing of this fusion

protein at a KEX-2 cleavage site at the junction between the α-mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

A 5' sense oligonucleotide of the following sequence and containing a HindIII site for cloning was synthesized:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC TTT GAC GTG GAG AGG (SEQ ID NO: 42)

A 3' antisense oligonucleotide of the following sequence and containing both a BamHI site for cloning and a stop codon was synthesized:

CGC GGA TCC CTA CTG GCG GAA GCA GCG GAG CAT GCA GGC CTC CTC AGA GCG GTA GCT GTT CTT ATT GCC CCG GCA GCC TCC ATA GAT GAA GTT ATT GCA GGA GTT CCT CTC CAC GTC AAA GTA CCA GCG (SEQ ID NO: 43)

The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII and BamHI. The resulting digested double stranded fragment was cloned into pUC19 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC TTT GAC GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC TAT GGA GGC TGC CGG GGC AAT AAG AAC AGC TAC CGC TCT GAG GAG GCC TGC ATG CTC CGC TGC TTC CGC CAG TAG GGA TCC (SEQ ID.: 44)

which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transf rmed into yeast strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were

pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159).

Detection of expression of placental bikunin (102-159) in transformed yeast

Firstly, the supernatants (50 ul per assay) were evaluated for their capacity to inhibit the *in vitro* activity of trypsin using the assay methods as described in Example 1 (1 ml assay volume). An un-used media only sample as well as a yeast clone expressing an inactive variant of aprotinin served as negative controls. A yeast clone expressing natural aprotinin served as a positive control and is shown for comparison.

The second method to quantify placental bikunin (102-159) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug of purified reduced synthetic placental bikunin (102-159), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 µl) wer subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's directions (Kirkegaard and Perry, Gaithersburg, MD).

Purification of placental bikunin (102-159) from a transformed strain of SC101

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM $CaCl_2$ and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted fractions were pooled and applied to a C18 column (Vydac, 5um, 4.6 x 250 mm) previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin (102-159) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

Results. Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159). The same 6 kDa band in the aprotinin control did not react with the same antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal

sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) while about 60 % of the material contained a portion of the yeast α-mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent Ki of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) in fermentation broth as well as the isolation of placental bikunin (102-159) from one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159, as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (YEEY–) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P' subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (defined as construct #1) but with the following modifications:

Construct #2 placental bikunin 103-159, yeast codon usage

A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG CTAATGCTGT TACTGGTCCA TGTAGAGCTT CTTTTCCAAG ATGGTACTTT GATGTTGAAA GA (SEQ ID NO: 55)

and 3' antisense oligonucleotide

ACTGGATCCT CATTGGCGAA AACATCTCAA CATACAGGCT TCTTCAGATC TGTAAGAATT TTTATTACCT CTACAACCAC CGTAAATAAA ATTATTACAA GAATTTCTTT CAACATCAAA GTACCATCT (SEQ ID NO: 56)

were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159

Construct #3 placental bikunin 101-159, yeast codon usage
A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT GTACTGCTAA TGCTGTTACT GGTCCATGTA GAGCTTCTTT TCCAAGATGG TACTTTGATG TTGAAAGA (SEQ ID NO: 57)

and the same 3' antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159.

Construct #4 placental bikunin 98-159, yeast codon usage
A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGATATG TTTAATTACG AAGAATACTG TACTGCTAAT GCTGTTACTG GTCCATGTAG AGCTTCTTTT CCAAGATGGT ACTTTGATGT TGAAAGA (SEQ ID NO: 58)

and the same 3' antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above).

Yeast strain SC101 (MATα, ura 3-52, suc 2) was transformed with the plasmids containing each of the above cDNAs, and proteins were expressed using the methods that were described above for the production of placental bikunin 102-159 with human codon usage. Approximately 250 ml of each yeast culture was harvested, and the supernatant from centrifugation (15 min x 3000 RPM) separately subjected to purification over 1 ml columns of kallikrein-sepharose as described above. The relative amount of trypsin inhibitory activity in the applysate, the amount of purified protein recovered and the N-terminal sequence of the purified protein were determined and are listed below in Table 7.

Table 7
Relative production levels of different proteins containing the C-terminal Kunitz domain of placental bikunin

TABLE 7

Construct		Relative conc. of inhibitor in applysate	N-terminal sequencing amount (pmol)	sequence	Comments
#2	103-159	none detected	none	none	no expression
#3	101-159	25 % inhibition	none	none	low expression
#4	98-159	93 % inhibition	910	DMFNYE-	good expression correct product
#1	102-159	82 % inhibition	480	AKEEGV-	expression of active incorrectly processed protein

The results show that placental bikunin fragments of different lengths that contain the C-terminal Kunitz domain show wide variation in capacity to express functional secreted protein. Constructs expressing fragments 101-159 and 103-159 yielded little or low enzymic activity in the supernatants prior to purification, and N-terminal sequencing of 0.05 ml aliquots of each purified fraction yielded undetectable amounts of inhibitor. On the other hand expression either of placental bikunin 102-159 or 98-159 yielded significant amounts of protease activity prior to purification. N-terminal sequencing however showed that the purified protein recovered from expression of 102-159 was once again largely incorrectly processed, exhibiting an N-terminus consistent with processing of the majority of the pre-protein at a site within the yeast α-mating factor pro-sequence. The purified protein recovered from expression of placental bikunin 98-159 however was processed entirely at the correct site to yield the correct N-terminus. Furthermore, nearly twice as much protein was recovered as compared to the recovery of placental bikunin 102-159. Placental bikunin 98-159 thus represents a preferred fragment length for the production of the C-terminal Kunitz domain of placental bikunin by the α-mating factor pre-pro sequence/ KEX-2 processing system of *S. cerevisiae*,

Example 6

Alternative procedure for yeast expression

The 58 amino acid peptide derived from the R74593 translation product can also be PCR amplified from either the R87894-R74593 PCR product cloned into the TA vector™ (Invitrogen, San Diego, CA) after DNA sequencing or from human placental cDNA. The amplified DNA product will consist of 19 nucleotides from the yeast α-mating factor leader sequence mated to the R74593 sequence which codes for the YEEY--CFRQ (58 residues) so as to make the translation product in frame, constructing an α -mating factor/Kunitz domain fusion protein. The protein sequence also contains a kex 2 cleavage which will liberate the Kunitz domain at its native N-terminus.

The 5' sense oligonucleotide which contains a HindIII site for cloning will contain the following sequence:

GCCAAGCTTG GATAAAAGAT ATGAAGAAT ACTGCACCGC CAACGCA (SEQ ID NO: 30)

The 3' antisense oligonucleotide contains a BamHI site for cloning as well as a stop codon and is of the following sequence:

GGGGATCCTC ACTGCTGGCG GAAGCAGCGG AGCAT (SEQ ID NO: 31)

The full 206 nucleotide cDNA sequence to be cloned into the yeast expression vector is of the following sequence:

CCAAGCTTGG ATAAAAGATA TGAAGAATAC TGCACCGCCA ACGCAGTCAC TGGGCCTTGC CGTGCATCCT TCCCACGCTG GTACTTTGAC GTGGAGAGGA ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGTGAGG ATCCCC (SEQ ID NO: 32)

After PCR amplification, this DNA will be digested with HindIII, BamHI and cloned into the yeast expression vector pMT15 (see US patent 5,164,482, incorporated by reference in the entirety) also digested with HindIII and BamHI. The resulting plasmid vector is used to transform yeast strain SC 106 using the methods described in US patent 5,164,482. The URA 3+ yeast transformants are isolated and cultivated under inducing conditions. The yield of recombinant Placental bikunin variants is determined according to the

amount of trypsin inhibitory activity that accumulated in the culture supernatants over time using the *in vitro* assay method described above. Fermentation broths are centrifuged at 9000 rpm for 30 minutes. Th supernatant is then filtered through a 0.4 then a 0.2 µm filter, diluted to a conductivity of 7.5 ms, and adjusted to pH 3 with citric acid. The sample is then batch absorbed onto 200 ml of S-sepharose fast flow (Pharmacia) in 50 mM sodium citrate pH 3 and stirred for 60 min. The gel is subsequently washed sequentially with 2 L of each of: 50 mM sodium citrate pH 3.0; 50 mM Tris-HCL pH 9.0; 20 mM HEPES pH 6.0. The washed gel is transferred into a suitable column and eluted with a linear gradient of 0 to 1 M sodium chloride in 20 mM HEPES pH 6.0. Eluted fractions containing *in vitro* trypsin inhibitory activity are then pooled and further purified either by a) chromatography over a column of immobilized anhydrotrypsin (essentially as described in Example 2); b) by chromatography over a column of immobilized bovine kallikrein; or c) a combination of conventional chromatographic steps including gel filtration and/or anion-exchange chromatography.

Example 7
Isolation and characterization of native human placental bikunin from placenta

Bikunin protein was purified to apparent homogeniety from whole frozen placenta (Analytical Biological Services, Inc, Wilmington, DE). The placenta (740 gm) was thawed to room temperature and cut into 0.5 to 1.0 cm pieces, placed on ice and washed with 600 ml PBS buffer. The wash was decanted and 240 ml of placenta pieces placed into a Waring blender. After adding 300 ml of buffer consisting of 0.1 M Tris (pH 8.0), and 0.1 M NaCl , the mixture was blended on high speed for 2 min, decanted into 750.0 ml centrifuge tubes, and placed on ice. This procedure was repeated until all material was processed. The combined slurry was centrifuged at 4500 x g for 60 minutes at 4°C. The supernatant was filtered through cheese cloth and the placental bikunin purified using a kallikrein affinity column made by covalently attaching 70 mg of bovine pancreatic kallikrein (Bayer AG) to 5.0 mls of CNBr activated Sepharose (Pharmacia) according to manufacturers instruction. The material was loaded onto the affinity column at a flow rate of 2.0 ml/min and washed with 0.1 M Tris (pH 8.0), 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was further washed with 0.1 M Tris (pH 8.0), 0.5 M NaCl and then eluted with 3 volumes of 0.2 M acetic acid, pH 4.0. Fractions containing kallikrein and trypsin inhibitory (see below)

activity were pooled, frozen, and lyophilized. Placental bikunin was further purified by gel-filtration chromatography using a Superdex 75 10/30 (Pharmacia) column attached to a Beckman System Gold HPLC system. Briefly, the column was equilibrated in 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 at a flow rate of 0.5 ml/min. The lyophilized sample was reconstituted in 1.0 ml of 0.1 M Tris, pH 8.0 and injected onto the gel-filtration column in 200 µl aliquots. Fractions were collected (0.5 ml) and assayed for trypsin and kallikrein inhibitory activity. Active fractions were pooled, and the pH of the solution adjusted to 2.5 by addition of TFA. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 20% acetonitrile in 0.1 %TFA. Separation was achieved using a linear gradient of 20 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min over 50 minutes after an initial 20 minute wash at 20% acetonitrile in 0.1% TFA. Fractions (1ml) were collected and assayed for trypsin and kallikrein inhibitory activity. Fractions containing inhibitory activity were concentrated using a speed-vac concentrator (Savant) and subjected to N-terminal sequence analysis.

Functional assays for Placental Bikunin:

Identification of functional placental bikunin was achieved by measuring its ability to inhibit bovine trypsin and human plasma kallikrein. Trypsin inhibitory activity was performed in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2.0 mM CaCl2, 0.1% Triton x-100) at room temperature in a 96-well microtiter plate (Perkin Elmer) using Gly-Pro-Lys-Aminomethylcoumarin as a substrate. The amount of coumarin produced by trypsin was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter equipped with a plate reader. Trypsin (23 µg in 100 µl buffer) was mixed with 20 µl of the sample to be tested and incubated for 10 minutes at 25°C. The reaction was started by the addition of 50 µl of the substrate GPK-AMC (33 µM final) in assay buffer. The fluorescence intensity was measured and the % inhibition for each fraction was determined by:

$$\% \text{ inhibition} = 100 \times [1 - Fo/F1]$$

where Fo is the fluorescence of the unknown and F1 is the fluorescence of the trypsin only control. Kallikrein inhibitory activity of the fractions was similarly measured using 7.0 nM kallikrein in assay buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 0.1% triton x-100) and 66.0 µM Pro-Phe-Arg-AMC as a substrate.

Determination of the *in vitro* specificity of placental bikunin

The *In vitro* specificity of native human placental bikunin was determined using the materials and methods as described in the preceding examples above. Placental bikunin was quantified by active site titration against a known concentration of trypsin using GPK-AMC as a substrate to monitor the fraction of unbound trypsin.

Protein Sequencing

The 1 ml fraction (C18-29 Delaria) was reduced to 300 ml in volume, on a Speed Vac, to reduce the amount of organic solvent. The sample was then loaded onto a Hewlett-Packard miniature biphasic reaction column, and washed with 1 ml of 2% trifluoroacetic acid. The sample was sequenced on a Hewlett-Packard Model G1005A protein sequencing system using Edman degradation. Version 3.0 sequencing methods and all reagents were supplied by Hewlett-Packard. Sequence was confirmed for 50 cycles.

Results. Placental Bikunin was purified to apparent homogeniety by sequential kallikrein affinity, gel-filtration, and reverse-phase chromatography (see purification table below):

Table 5
Purification table for native Placental Bikunin (1-179)

TABLE 5

Step	Vol (ml)	OD 280 (/ml)	OD 280	Units[a] (U)	Units/OD 280
Placenta Supernatant	1800.0	41.7	75,060	3,000,000	40.0
Kallikrein Affinity pH 4.0	20.0	0.17	3.36	16,000	4,880
Kallikrein Affinity pH 1.7	10.2	0.45	4.56	12,000	2,630
Superdex 75	15.0	0.0085	0.13	3,191	24,546

[a]One Unit is defined as that amount which inhibits 50% of trypsin activity in a standard assay.

The majority of the kallikrein and trypsin inhibitory activity eluted from the kallikrein affinity column in the pH 4.0 elution. Subsequent gel-filtration chromatography (Figure 5) yielded a peak of kallikrein and trypsin inhibitory activity with a molecular weight range of 10 to 40 kDa as judged by a standard

curve generated by running molecular weight standards under identical conditions. Reverse-phase C18 chromatography (Figure 6) yielded 4 peaks of inhibitory activity with the most potent eluting at approximately 30 % acetonitrile. The activity associated with the first peak to elute from C18 (fraction 29) exhibited an amino acid sequence starting with amino acid 1 of the predicted amino acid sequence of placental bikunin (ADRER...; SEQ ID NO: 1), and was identical to the predicted sequence for 50 cycles of sequencing (underlined amino acids in Figure 3). Cysteine residues within this sequence stretch were silent as expected for sequencing of oxidized protein. The cysteine residues at amino acid positions 11 and 20 of mature placental bikunin were later identified from sequencing of the S-pyridylethylated protein whereupon PTH-pyridylethyl-cysteine was recovered at cycles 11 and 20.

Interestingly, the asparagine at amino acid residue number 30 of the sequence (Figure 3) was silent showing that this site is likely to be glycosylated. Fraction 29 yielded one major sequence corresponding to that of placental bikunin starting at residue #1 (27 pmol at cycle 1) plus a minor sequence (2 pmol) also derived from placental bikunin starting at residue 6 (SIHD...). This shows that the final preparation sequenced in fraction 29 is highly pure, and most likely responsible for the protease inhibitory activity associated with this fraction (Figure 6).

Accordingly, the final preparation of placental bikunin from C18 chromatography was highly pure based on a silver-stained SDS-PAGE analysis (Figure 7), where the protein migrated with an apparent Mr of 24 kDa on a 10 to 20 % acrylamide tricine gel (Novex, San Diego, CA) calibrated with the following molecular weight markers: insulin (2.9 kDa); bovine trypsin inhibitor (5.8 kDa); lysozyme (14.7 kDa); β-lactaglobulin (18.4 kDa); carbonic anhydrase (29 kDa); and ovalbumin (43 kDa). The above size of placental bikunin on SDS-PAGE is consistent with that predicted from the full length coding sequence (Figure 4F).

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-159) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had

undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (Trasylol®).

Table 6
Ki values for the inhibition of various proteases by placental bikunin

TABLE 6

Protease (concentration)	Placental Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.13	0.8
Human Plasmin (50 pM)	1.9	1.3

The results show that placental bikunin isolated from a natural source (human placenta) is a potent inhibitor of trypsin-like serine proteases.

Example 8
Expression pattern of placental bikunin amongst different human organs and tissues

A multiple tissue northern was purchased from Clontech which contained 2 μg of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using ^{32}P-dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

Results. The pattern of tissue expression observed using a placental bikunin (102-159) probe (Figure 11A) or a larger probe containing both Kunitz

domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

The broad tissue distribution of the mRNA shows that placental bikunin is broadly expressed. Since the protein also contains a leader sequence it would have ample exposure to the human immune system, requiring that it become recognized as a self protein. Additional evidence for a broad tissue distribution of placental bikunin mRNA expression was derived from the fact that some of the EST entries with homology to placental bikunin (Figure 4B) were derived from human adult and infant brain, and human retina, breast, ovary, olfactory epithelium, and placenta. It is concluded therefore that administration of the native human protein to human patients would be unlikely to elicit an immune response.

Interestingly, the expression pattern of placental bikunin is somewhat reminiscent of that for bovine aprotinin which is found in high levels in bovine lung and pancreas. To further elucidate the expression pattern of placental bikunin, RT-PCR of total RNA from the following human cells was determined: un-stimulated human umbilical vein endothelial cells (HUVECs), HK-2 (line derived from kidney proximal tubule), TF-1 (erythroleukemia line) and phorbolester (PMA)-stimulated human peripheral blood leukocytes. The probes used:

CACCTGATCGCGAGACCCC (sense; SEQ ID NO: 59);
CTGGCGGAAGCAGCGGAGCATGC (antisense; SEQ ID NO: 60),

were designed to amplify a 600 b.p placental bikunin encoding cDNA fragment. Comparisons were normalized by inclusion of actin primers to amplify an 800 b.p. actin fragment. Whereas the 800 b.p fragment identified on agarose gels with ethidium bromide was of equal intensity in all lanes, the 600 b.p. placental bikunin fragment was absent from the HUVECs but present in significant amounts in each of the other cell lines. We conclude that placental bikunin is not expressed in at least some endothelial cells but is expressed in some leukocyte populations.

Example 9

Purification and properties of Placental Bikunin (1-170) highly purified from a Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector (see previous Examples) was liberated by digestion with HindIII and Xba1 yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a Pst1 site 3' to the XbaI site at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

5' CGC GTC TCG GCT GAC CTG GCC CTG CAG ATG GCG CAC GTG TGC GGG 3' (SEQ ID NO: 61)

A stop codon (TAG) and BglII / XmaI site was similarly engineered at the 3' end of the cDNA using the oligonucleotide:

5' CTG CCC CTT GGC TCA AAG TAG GAA GAT CTT CCC CCC GGG GGG GTG GTT CTG GCG GGG CTG 3' (SEQ ID NO: 62).

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with Pst1 and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) which contains both Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane segment.

The expression of Bikunin by Sf-9 insect cells was optimal at a multiplicity of infection of 1 to 1 when the medium was harvested at 72 h post infection. After harvesting, the baculovirus cell culture supernatant (2L) was adjusted to pH 8.0 by the addition of Tris-HCl. Bikunin was purified by chromatography using a 5 ml bovine pancreatic kallikrein affinity column as previously described in Example 7 for

the purification of native placental bikunin from placenta. Eluted material was adjusted to pH 2.5 with TFA and subjected to chromatography on a C18 reverse-phase column (1.0 x 25 cm) equilibrated in 10% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The bikunin was eluted with a linear gradient of 10 to 80% acetonitrile in 0.1% TFA over 40 min. Active fractions were pooled, lyophilized, redissolved in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 2 mM CaCl2, and 0.1% triton x-100, and stored at -20°C until needed. The concentration of recombinant bikunin was determined by amino acid analysis.

Results. Recombinant bikunin was purified from baculovirus cell culture supernatant using a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 8 below).

Table 8
Purification of recombinant bikunin from transformed culture supernatant

TABLE 8

Purification Step	Vol (ml)	OD 280/ml	OD 280 total	Units (U)	Specific activity (U/OD)
Supernatant	2300.0	9.0	20,700	6,150,000	297
Kallikrein affinity	23.0	0.12	2.76	40,700	14,746
C18 reverse-phase	0.4	3.84	1.54	11,111	72,150

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....) , showing that the signal peptide was correctly processed in Sf9 cells.

Purified placental bikunin from Sf9 cells (100 pmol) was pyridyl thyl-alkylated, CNBr digested and then sequenced without resolution of the resulting fragments. Sequencing for 20 cycles yielded the following N-terminii:

Sequence	Amount	Placental bikunin residue #
LRCFrQQENPP-PLG-----	21 pmol	154 - 168 (SEQ ID NO: 63)
ADRERSIHDFCLVSKVVGRC	20 pmol	1 - 20 (SEQ ID NO: 64)
FNYeEYCTANAVTGPCRASF	16 pmol	100 - 119 (SEQ ID NO: 65)
Pr--Y-V-dGS-Q-F-Y-G	6 pmol	25 - 43 (SEQ ID NO: 66)

Thus N-terminii corresponding to each of the expected four fragments were recovered. This confirms that the Sf9 expressed protein contained the entire ectodomain sequence of placental bikunin (1-170). N-terminal sequencing (50 cycles) of an additional sample of undigested Placental Bikunin (1-170) resulted in an amino acid sequence which at cycle 30 was devoid of any PTH-amino acid (PTH-asparagine was expected). A similar result was obtained upon sequencing of the natural protein from human placenta (Example 7) and is consistent with this residue being glycosylated as predicted from the amino acid sequence surrounding this asparagine residue. Furthermore, the cysteine residues within this region were also silent consistent with their participation in disulfide bonding.

Example 10

Inhibition specificity of purified placental bikunin derived from Sf9 cells.

The *in vitro* specificity of recombinant bikunin was determined using the materials and methods as described in Examples 3, 4 and 7. In addition, the inhibition of human tissue kallikrein by bikunin was measured by the incubation of 0.35 nM human tissue kallikrein recombinant bikunin in buffer containing 50 mM Tris (pH 9.0), 50 mM NaCl, and 0.01% triton x-100. After 5 min. at 37°C, 5 µl of 2 mM PFR-AMC was added and the change in fluorescence monitored.

Inhibition of tissue plasminogen activator (tPA) was also determined as follows: tPA (single chain form from human melanoma cell culture from Sigma Chemical Co, St Louis, MO) was pre-incubated with inhibitor for 2 hr at room temperature in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl, and 0.02% sodium azide. Reactions were subsequently initiated by transfer to a reaction system comprising the following initial component concentrations: tPA (7.5 nM), inhibitor 0 to 6.6 µM, DIle-Lpro-Larg-pNitroaniline (1mM) in 28 mM Tris buffer pH 8.5 containing 0.004 % (v/v) triton x-100 and 0.005% (v/v) sodium azide. Formation of p-Nitroaniline was determined from the A405nm measured following incubation at 37 C for 2hr.

The table below show the efficacy of recombinant bikunin as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for

screening inhibition using either recombinant bikunin, or aprotinin.

Table 9
Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) or aprotinin

TABLE 9

Protease (concentration)	Recombinant Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.064	0.8
Human Plasma Kallikrein (2.5 nM)	0.18	19.0
Human Tissue Kallikrein (0.35 nM)	0.04	0.004
Bovine Pancreatic Kallikrein (100 pM)	0.12	0.02
Human Plasmin (50 pM)	0.23	1.3
factor Xa (0.87 nM)	180	5% Inhibition at 31 µM
factor XIa (0.1 nM)	3.0	288
tissue plasminogen activator (7.5 nM)	< 60	no inhibition at 6.6 µM
Tissue Factor VIIa	800	no inhibition at 1 µM

The results show that recombinant bikunin can be expressed in insect cells to yield an active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant bikunin was more potent that the synthetically derived bikunin fragments (7-64) and (102-159) against all enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Besides measuring the potencies against specific proteases, the capacity of placental bikunin (1-170) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra^{R} 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N.Y.). The instrument was set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl2 were automatically dispensed to initiate clotting, and the clotting time was monitored automatically. The results

(Figure 14) showed that a doubling of the clotting time required approximately 2 μM final aprotinin, but only 0.3 μM Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and usefull as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

Although certain embodiments of the invention have been described in detail for the purpose of illustration, it will be readily apparent to those skilled in the art that the methods and formulations described herein may be modified without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WE CLAIM:

1. A substantially purified protein, having serine protease inhibitory activity, selected from the group of proteins consisting of materials each of which comprises one of the following amino acid sequences, the amino acids of said sequences being numbered in accordance with the amino acid sequence of native human placental bikunin shown in figure 4F in which the N-terminal residue generated by removal of signal peptide is designated as residue 1:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN      50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF     100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE     150
ACMLRCFRQQ ENPPLPLGSK                                      170
(SEQ ID NO: 52);

MAQLCGL RRSRAFLALL GSLLLSGVLA                               -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN      50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF     100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE     150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN     200
QERALRTVWS SGDDKEQLVK NTYVL                                225
(SEQ ID NO: 49);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN      50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF     100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE     150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN     200
QERALRTVWS SGDDKEQLVK NTYVL                                225
(SEQ ID NO: 70);

    AGSFLAWL GSLLLSGVLA  -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN      50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF     100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE     150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS                            179
(SEQ ID NO: 2);
```

MLR AEADGVSRLL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS SGDDKEQLVK NTYVL 225
(SEQ ID NO: 45);

MAQLCGL RRSRAFLALL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 47);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 71);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATV 64
(SEQ ID NO: 4);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK C 61
(SEQ ID NO: 5);

YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6);

CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE 150
ACMLRC 156

(SEQ ID NO: 7);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQ 159
(SEQ ID NO: 3);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRC 156
(SEQ ID NO: 50);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 25
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 1); and

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS 92
(SEQ ID NO: 8).

2. A protein as in claim 1, wherein said protein is glycosylated, or contains at least one intra-chain cysteine-cysteine disulfide bond, or is both glycosylated and contains at least one intra-chain cysteine-cysteine disulfide bond.

3. A pharmaceutical composition for inhibiting serine protease activity, comprising a protein of claim 1 or claim 2 plus a pharmaceutically acceptable carrier.

4. An isolated nucleic acid sequence which encodes for a protein of claim 1.

5. A self-replicating protein expression vector containing a nucleic acid sequence which encodes for and is capable of expressing a protein of claim or claim 2.

6. A method for inhibiting serine protease activity comprising contacting serine protease with an effective amount of at least one protein of claim 1 or claim 2.

7. A method for treating a condition of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, polytrauma, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis comprising administering to a subject having such a condition an effective amount of the protein of claim 1 or claim 2.

8. The method of Claim 7 wherein said condition is brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, or open heart surgery.

9. The method of Claim 7 wherein said condition is gastric cancer, cervical cancer, or prevention of metastasis.

10. A method for the preparation of a medicament for the treatment of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis.

11. A method for preparing a protien of claim 1 or claim 2 using recombinant DNA technology.

FIGURE 1

```
R35464 GGCCGGGTCG TTTCTCGCCT GGCTGGGATC GCTGCTCCTC TCTGGGGTCC   50
ORF      P  G  R  F  S  P  G  W  D  R  C  S  S  L  G  S      16

R35464 TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  100
ORF    W  P  A  D  R  E  R  S  I  H  D  F  C  L  V  S  K     33

R35464 GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA  150
ORF      V  V  G  R  E  R  A  S  M  P  R  W  W  Y  N  V  T    50

R35464 CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC  200
ORF      D  G  S  C  Q  L  F  V  Y  G  G  C  D  G  N  S      66

R35464 AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  250
ORF    N  N  Y  L  T  K  E  E  C  L  K  K  C  A  T  V  T     83

R35464 AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT  300
ORF      E  N  A  T  G  D  L  A  T  S  R  N  A  A  D  S  S  100

R35464 CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA  350
ORF      V  P  S  A  P  R  R  Q  D  S  *  R  P  L  Q  R     116

R35464 TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT-------  393
ORF    Y  V  S  *  I  *  R  I  I  A  P  *  T  *              130
```

KEY
R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO: 12)
ORF = EST R35464 Open Reading Frame Translation (SEQ ID NO: 13)

FIGURE 2

```
R74593 GCAATAATTA CCTGACCAAG GAGGAGTGCC TCAAGAAATG TGCCACTGTC   50
ORF      Q  *  L  P  D  Q  G  G  V  P  Q  E  M  C  H  C  H    17

R74593 ACAGAGAATG CCACGGGTGA CCTGGCCACC AGCAGGAATG CAGCGGATTC  100
ORF      R  E  C  H  G  *  P  G  H  Q  Q  E  C  S  G  F      33

R74593 CTCTGTCCCA AGTCTCCCAG AAGGCAGGAT TCTGAAGACC ACTCCAGCGA  150
ORF    L  C  P  K  S  P  R  R  Q  D  S  E  D  H  S  S  D    50

R74593 TATGTTCAAC TATGAAGAAT ACTGCACCGC CAACGCAGTC ACTGGGCCTT  200
ORF      M  F  N  Y  E  E  Y  C  T  A  N  A  V  T  G  P  C   67

R74593 GCCGTGCATC CTTCCCACGC TGGTACTTTG ACGTGGAGAG GAACTCCTGC  250
ORF      R  A  S  F  P  R  W  Y  F  D  V  E  R  N  S  C      83

R74593 AATAACTTCA TCTATGGAGG CTGCCGGGGC AATAAGAACA GCTACCGCTC  300
ORF    N  N  F  I  Y  G  G  C  R  G  N  K  N  S  Y  R  S    100

R74593 TGAGGAGGCC TGCATGCTCC GCTGCTTCCG CCAGCAGGAG AATCCTCCCC  350
ORF      E  E  A  C  M  L  R  C  F  R  Q  Q  E  N  P  P  L  117

R74593 TGCCCCTTGG CTCAAAGGTG GTGGTTCTGG CCGGGGCTGT TTCGTGATGG  400
ORF      P  L  G  S  K  V  V  V  L  A  G  A  V  S  *  W    133

R74593 TGTTGATCCT TTTCCTGGGG AGCNTCCATG GTCTTACTGA TTCCGGGTGG  450
ORF    C  *  S  F  S  W  G  A  S  M  V  L  L  I  P  G  G   150

R74593 CAAGGAGGAA CCAGGAGCGT GCCCTGCGGA NCGTCTGGAG CTTCGGAGAT  500
ORF      K  E  E  P  G  A  C  P  A  X  R  L  E  L  R  R  *  167

R74593 GACAAGGGNT
ORF      Q  G                                               510
                                                            169
```

KEY
R74593 = Nucleic acid sequence of EST R74593 (SEQ ID NO: 14)
ORF = EST R74593 Open Reading Frame Translation (SEQ ID NO: 15)

FIGURE 3

```
R35464          GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
N39798                                 TGGGANTC GCTGCTCCTC TCTGGGGTCC  23
H94519           GCNGCG-CGT TNNTCGCNT- GCTGGGA-TC GCTGCACCTC TCTGGGGTCG  47
R74593 corr.     ---------- ---------- ---------- ---------- ----------
Consensus       GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
Translation      A  G  S  F     L  A  W     L  G     S     L  L  L     S  G  V      -3

R35464          TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA 100
N39798          TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  77
H94519          NGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  96
R74593 corr.    ---------- ---------- ---------- ---------- ----------
Consensus       TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  99
Translation      L  A  A  D     R  E  R     S  I  H     D  F  C  L     V  S  K     15

R35464          GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 150
N39798          GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 127
H94519          GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 146
R74593 corr.    ---------- ---------- ---------- ---------- ----------
Consensus       GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 149
Translation      V  V  G     R  C  R  A     S  M  P     R  W  W     Y  N  V  T     32

R35464          CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 200
N39798          CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 177
H94519          CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 196
R74593 corr.    ---------- ---------- ---------- ---------- --------GC   2
Consensus       CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 199
Translation       D  G  S     C  Q  L     E  V  Y  G     G  C  D     G  N  S      48

R35464          AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 250
N39798          AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 227
H94519          AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 246
R74593 corr.    AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  52
Consensus       AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 249
Translation     N  N  Y  L     T  K  E     E  C  L     K  K  C  A     T  V  T      65

R35464          AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 300
N39798          AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 277
H94519          AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 296
R74593 corr.    AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 102
Consensus       AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 299
Translation      E  N  A     T  G  D  L     A  T  S     R  N  A     A  D  S  S     82

R35464          CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA 350
N39798          CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 326
H94519          CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 345
R74593 corr.    CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 151
Consensus       CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 348
Translation       V  P  S     A  P  R     R  Q  D  S     E  D  H     S  S  D      98

R35464          TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT-------- 393
N39798          TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 372
H94519          TATGTT-CAA CTA-TG-AAG AATACTGGCA CCGCCAACGC ATTCACTGGG 392
R74593 corr.    TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 197
Consensus       TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 394
Translation      M  F     N     Y     E     E     Y  C     T     A  N  A     V  T  G     113
```

FIGURE 3 (CONT)

```
R35464          ---------- ---------- ---------- ---------- ----------
N39798          CCTTGC-GTG GAATCCTTTC CCACGCTGGN AATTTNGACG TTGAGAAGGA 421
H94519          CCT-GC-GTG -CATCCTT-C CCACGCTGGT ACTTT-GNCG ---------- 427
R74593 corr.    CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 243
Consensus       CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 440
Translation     P  C  R  A     S  F     P  R  W  Y     F  D  V     E  R     N 129

R35464          ---------- ---------- ---------- ---------- ----------
N39798          AC-------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ---------- 423
R74593 corr.    ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 293
Consensus       ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 490
Translation       S  C  N     N  F  I     Y  G  G  C     R  G  N     K  N  S     145

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 343
Consensus       TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 540
Translation     Y  R  S  E     E  A  C     M  L  R     C  F  R  Q     Q  E  N  162

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 393
Consensus       TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 590
Translation       P  P  L     P  L  G  S     K  V  V     V  L  A     G  A  V  S 179

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 443
Consensus       CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 640
Translation       *  W  C     *  S  F     S  W  G  A     S  M  V     L  L  I     195

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 493
Consensus       CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 690
Translation     P  G  G  K     E  E  P     G  A  C     P  A  *  R     L  E  L     212

R35464          ---------- --------
N39798          ---------- --------
H94519          ---------- --------
R74593 corr.    TCGGAGATGA CAAGGGNT                                          511
Consensus       TCGGAGATGA CAAGGGNT                                          708
Translation     R  R  *     Q  G                                             217
```

KEY

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO.: 12)
N39798 = Nucleic acid sequence of EST N39798 (SEQ ID NO.: 17)
H94519 = Nucleic acid sequence of EST H94519 (SEQ ID NO.: 16)
R74593 corr. = Corrected version of (SEQ ID NO.: 14) G at b.p. 114
Consensus = Nucleic acid sequence for human bikunin (SEQ ID NO.: 9)
Translation = Amino acid Translation of Consensus (SEQ ID NO.: 10)

Figure 4 A.

Schematic depicting the overlap of ESTs bearing homology to the cDNA sequence encoding placental bikunin

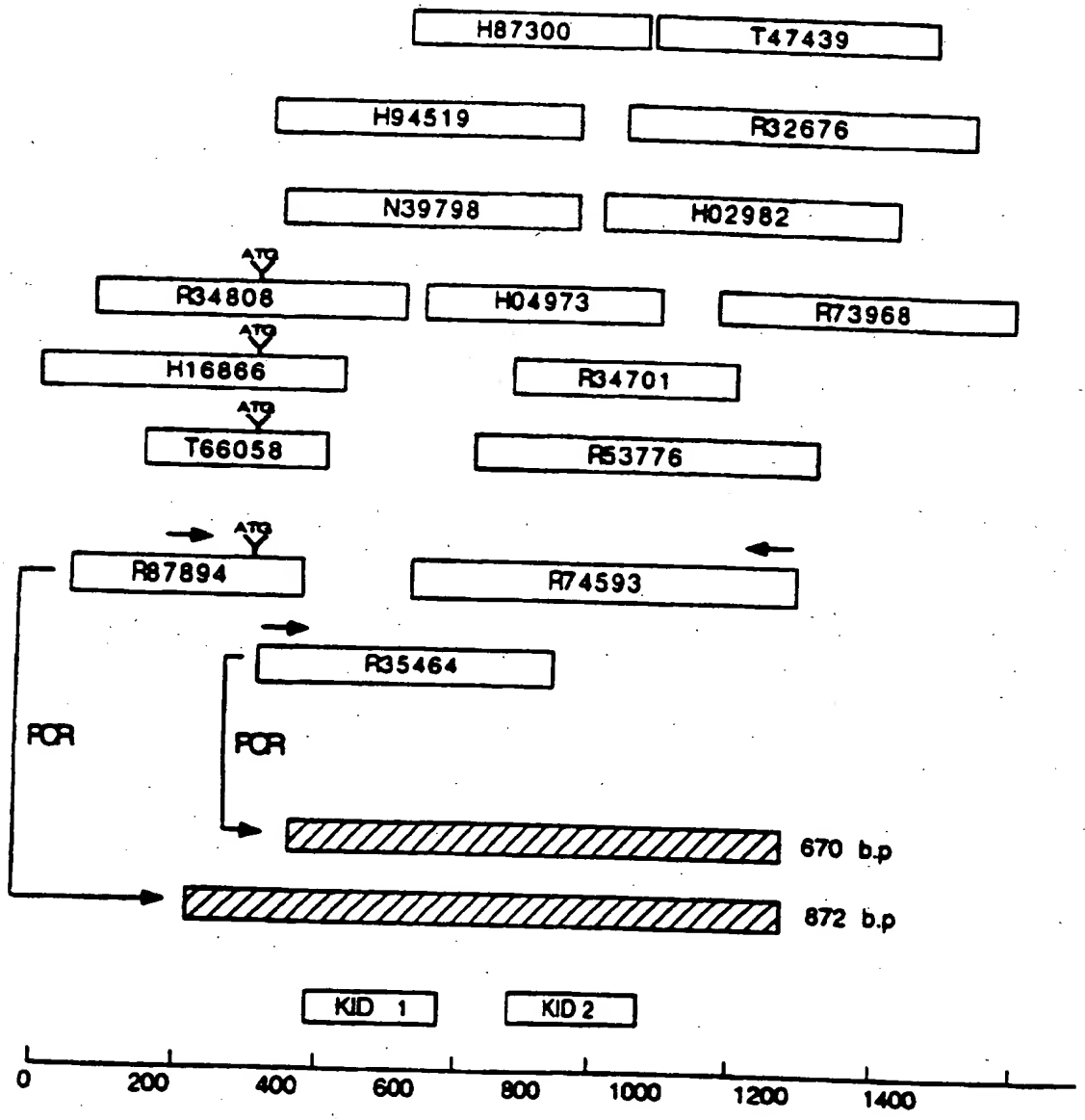
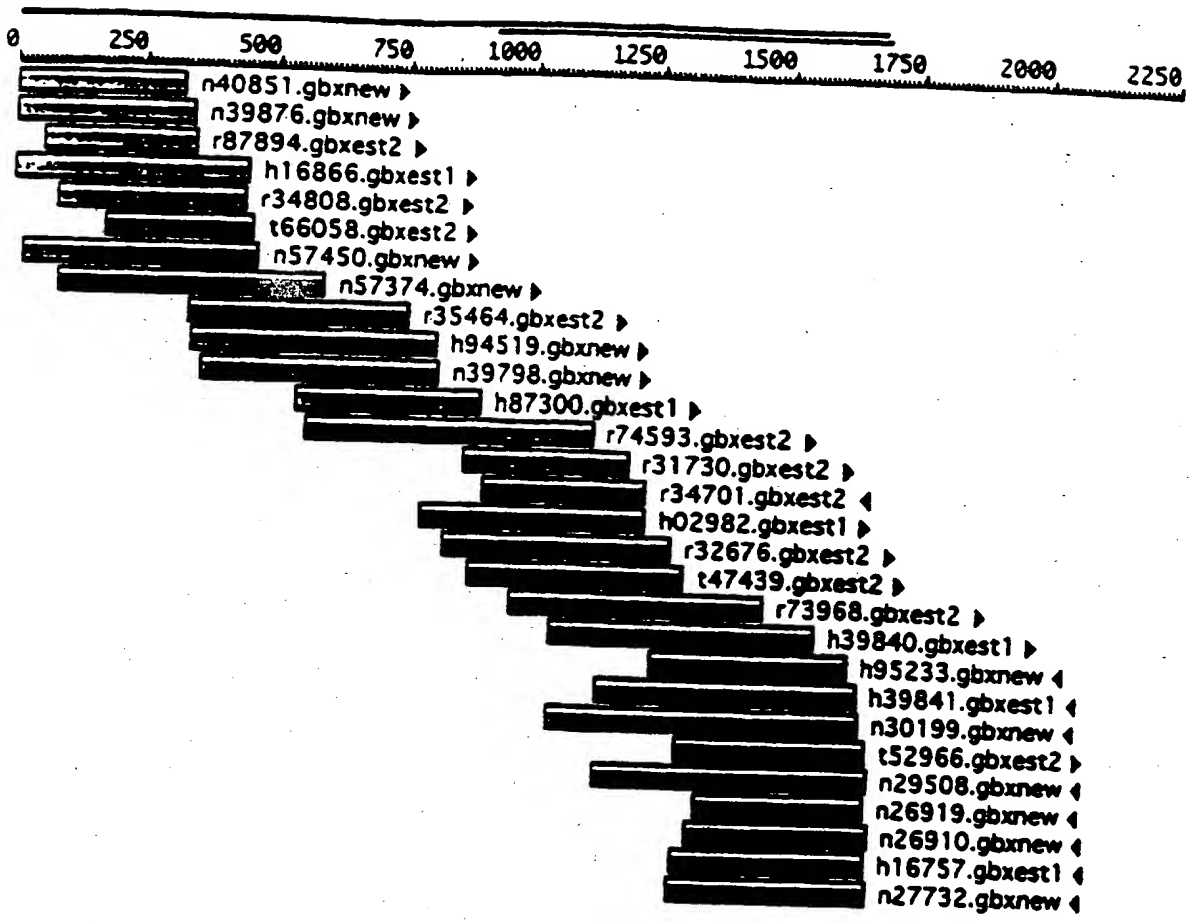


Figure 4B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500
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r87894.gbxest2
h16866.gbxest1
r34808.gbxest2
t66058.gbxest2
n57450.gbxnew
n57374.gbxnew
r35464.gbxest2
h94519.gbxnew
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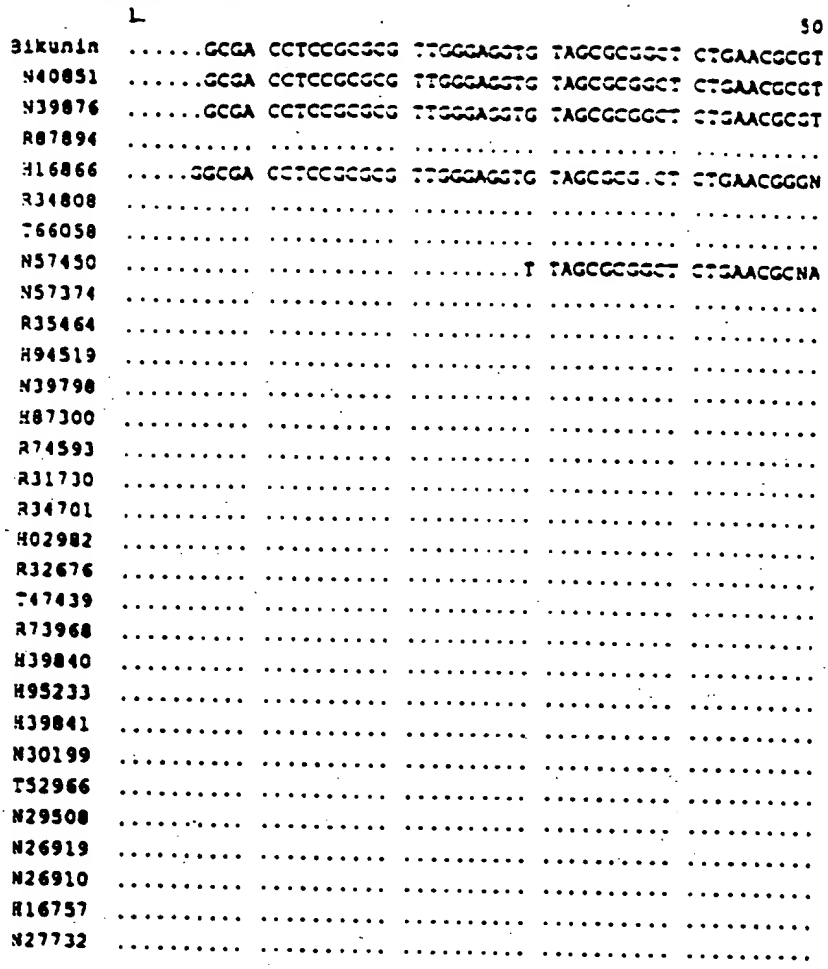
h16757.gbxest1
n27732.gbxnew

Figure 4C


1
50
Bikunin .....GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N40851 .....GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N39876 .....GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
R87894 .....
H16866 .....GGCGA CCTCCGCGCG TTGGGAGGTG TAGCGCG.CT CTGAACGGGN
R34808 .....
T66058 .....
N57450 .....T TAGCGCGGCT CTGAACGCNA
N57374 .....
R35464 .....
H94519 .....
N39798 .....
H87300 .....
R74593 .....
R31730 .....
R34701 .....
H02982 .....
R32676 .....
T47439 .....
R73968 .....
H39840 .....
H95233 .....
H39841 .....
N30199 .....
T52966 .....
N29508 .....
N26919 .....
N26910 .....
H16757 .....
N27732 .....

Figur 4C (C n't)

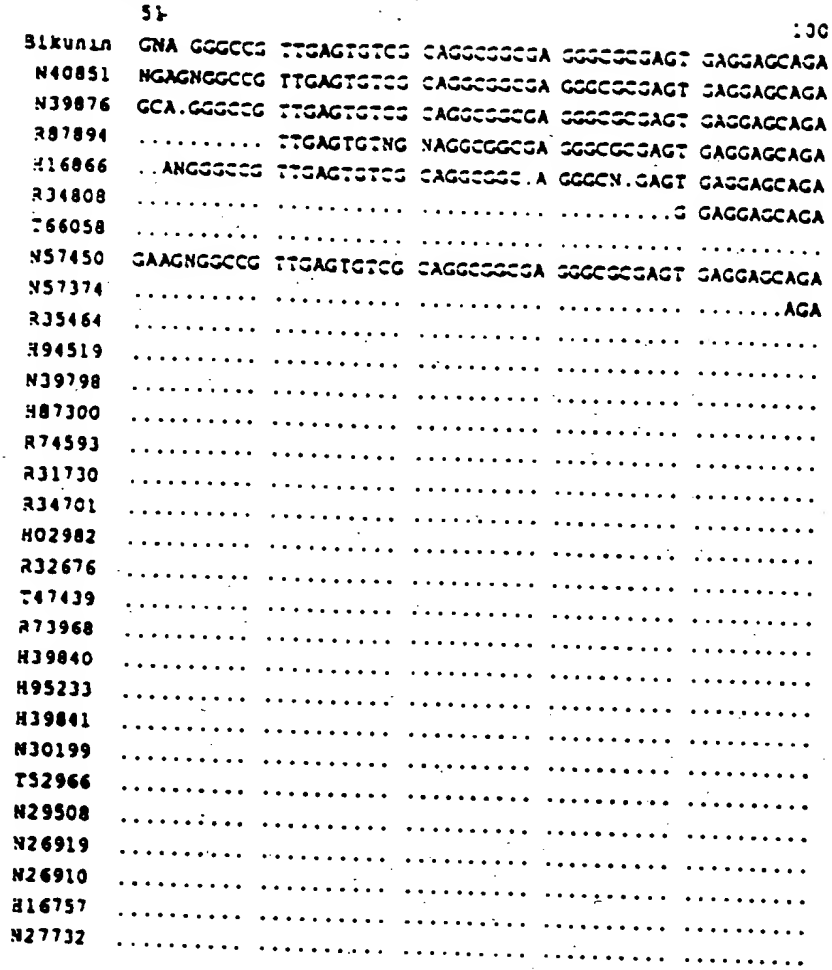

51
100
Blkunin GNA GGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N40851 NGAGNGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N39876 GCA.GGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
R87894 .... TTGAGTGTNG NAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
H16866 ..ANGGGCCG TTGAGTGTCG CAGGCGGC.A GGGCN.GAGT GAGGAGCAGA
R34808 ....G GAGGAGCAGA
T66058 ....
N57450 GAAGNGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N57374 ....AGA
R35464 ....
H94519 ....
N39798 ....
H87300 ....
R74593 ....
R31730 ....
R34701 ....
H02982 ....
R32676 ....
T47439 ....
R73968 ....
H39840 ....
H95233 ....
H39841 ....
N30199 ....
T52966 ....
N29508 ....
N26919 ....
N26910 ....
H16757 ....
N27732 ....

Figure 4C (Con't)

```
          101                                                        150
Blkunin   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
N40851    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
N39876    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC NTCCCCACAC TGAAGGTCCG
R87894    CCCAGGCATC GCGCGCCGAG AAGGCCGGGC GTCCCCACAC TGAAGGTCCG
H16866    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
R34808    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
T66058    .......... .......... .......... .......... ..........
N57450    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
N57374    CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
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Figure 4C (C n't)

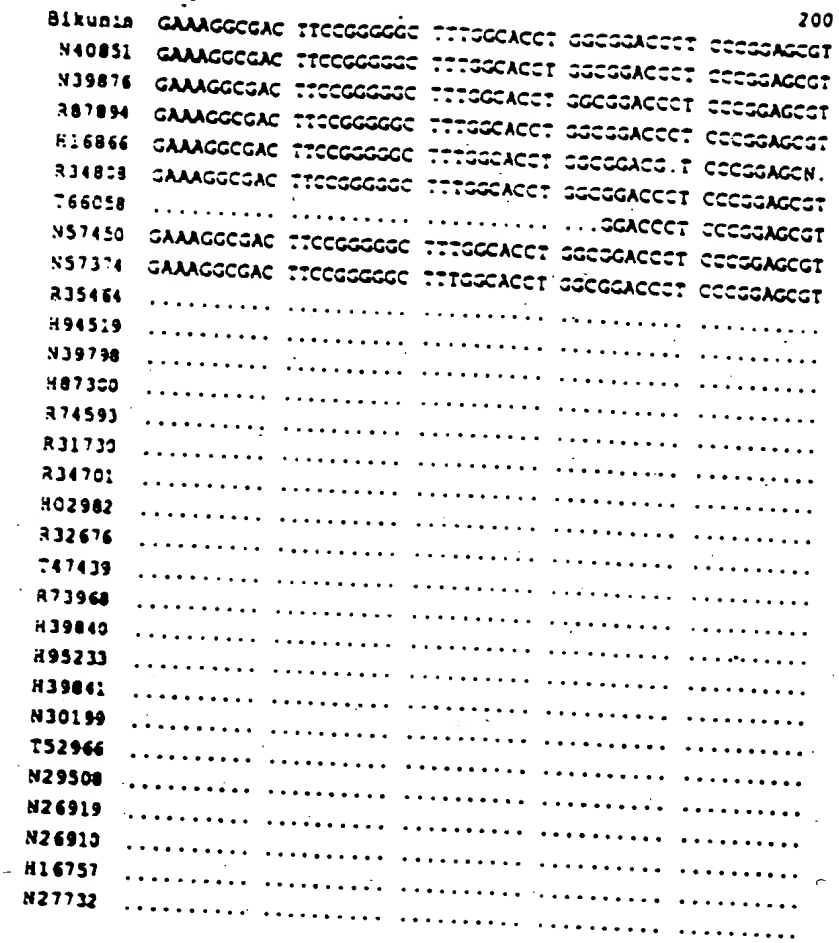

Figure 4C (C n't)

```
          201                                                        250
Bikunin   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
N40851    CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG .AGGGGCTTC
N39876    CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
R87894    CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
H16866    .GGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
R34808    CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG GAGGGGCTTC
T66058    CGGCACCTGA ACGCGAGGC. CTCCATTGCG .GTGCGTGTG NAGGGGCTTC
N57450    CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
N57374    CGGCACCTGA ACGCGAGGC. CTCCATTGC. CGTGCGTTNG .AGGGGCTTC
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

```
          251                                                       300
Bikunin   CCGCACCT G ATCGCGAGAC CCCAACGGCT GGTGG CGTC GC TG CGCG
N40851    CCGCACCT.G ATCGCGAGAC CCCAACGGCT.GGTGG.CGTC GCCTG.CGCG
N39876    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
R87894    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TN.CGCG
H16866    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TGGCGCG
R34808    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGGGCGTC GC.TG.CGCG
T66058    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
N57450    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
N57374    CCGGAACTTG ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figur 4C (Con't)

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          301                                                        350
Blkunin   TC TCGGCTG AGCT GGCCA TGGCGCANT  GTTGC GGGC T GAGGC GG
N40851    TC.TCGGCTG AGCT.GGNCA TGTCG
N39876    TC.TCGGCTG AGCT.GGCCA TGGCGCACT. G.TGCGGNGC T.GAGGC.G
R87894    TC.TCGGCTG AGCTTGGCCA TGGCGCANT. GTTNC.GGGC T.NAGGC.GG
H16866    TTCTCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
R34808    TCTTCGGCTG AGCTGGGCCA TGGCGCANTT GTTGC.GGGC T.GAGGC.GG
T66058    TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
N57450    TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GGTGC.GGGC TTGAGGC.GG
N57374    TCGTCGGCTG AGCT.GGCCA TGGCGCANT. GGTGCCGNGC T.GAGGCCGG
R35464    .......... .......... .......... .......... ....GGCCGG
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          351                                                                 400
Bikunin   AC  GG CG    TTTCTCG  CC TGCTGGG A TCGCT GC T CCTCTCT
R87894    ACG.
H16866    AC..CGNCGT TTTTCTTCG. CCTTGCTGGG ATTCGCTTGC TTCCTNTCTG
R34808    ACCCGGNCG. .TTTTTCGN CCTTGCTGGG ATTCG.TTG. TTNCTCTCTN
T66058    ...CGGNCG. .TTTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57450    ANN.NGCCG. ..TTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57374    AG..GGCCGG ..TTTCTCG. CCTTGCTGGG A.TCGCT.GC T.CCTCTCT.
R35464    .....GTCG. ..TTTCTCG. CCTGGCTGGG A.TCGCT.GC T.CCTCTCTG
H94519    .GCNGCGCG. ..TTNNTCG. CN.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N39798    .......... .......... .....CTGGG ANTCGCT.GC T.CCTCTCT.
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

```
          401                                                                 450
Bikunin   GGGG TCCTG G  CGGCCGA CCGA GAACG CA GCA TCC ACGACTT CT
H16866    GGGGTTCCTG GG.CGGCCGA CCGA.GAACG CA.GCA.TCC AAGAATTTTT
R34808    GGGGTTC.TG GGGNGGCCGA NCGA.GAACG CAAGCA.TTC ACGA.TTT
T66058    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGANTT.CT
N57450    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N57374    GGGG.TCCTG G..CGGCCGA NCGAAGAANG CA.GCAATCC ANGAATTNCT
R35464    GGGG.TCCTG G.CGGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H94519    GGGG.TCGNG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N39798    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

```
          451                                                       500
Sikunin   GCCTGGTGT CGAAGGT GG TGGGCAGATG CCGGG CCTC CATGCCTA G
H16866    GCC
T66058    TCCTGGTGTT CGAAGG
N57450    GCCTGGTGT. CGAAGGT.GG TGGGCAG
N57374    GCCTGGTGTT CGAAAGTTGG TGGGCANATT CCGGGGCCTT CATGNCTAAG
R35464    GCCTGGTGT. CGAAGGT.GG TGGGCAGATT CCGGG.CCTC CATGCCTA.G
H94519    GCCTGGTGT. CGAAGGT.GG TGGGCAGATG CCGGG.CCTC CATGCCTA.G
N39798    GCCTGGTGT. CGAAGGT.GG TGGGCAGATG CCGGG.CCTC CATGCCTA.G
H87300    ..........  ..........  ..........  ..........  ..........
R74593    ..........  ..........  ..........  ..........  ..........
R31730    ..........  ..........  ..........  ..........  ..........
R34701    ..........  ..........  ..........  ..........  ..........
H02982    ..........  ..........  ..........  ..........  ..........
R32676    ..........  ..........  ..........  ..........  ..........
T47439    ..........  ..........  ..........  ..........  ..........
R73968    ..........  ..........  ..........  ..........  ..........
H39840    ..........  ..........  ..........  ..........  ..........
H95233    ..........  ..........  ..........  ..........  ..........
H39841    ..........  ..........  ..........  ..........  ..........
N30199    ..........  ..........  ..........  ..........  ..........
T52966    ..........  ..........  ..........  ..........  ..........
N29508    ..........  ..........  ..........  ..........  ..........
N26919    ..........  ..........  ..........  ..........  ..........
N26910    ..........  ..........  ..........  ..........  ..........
H16757    ..........  ..........  ..........  ..........  ..........
N27732    ..........  ..........  ..........  ..........  ..........
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Figure 4C (C n't)

```
          501                                                        550
Bikunin   G TGGT GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
N57374    GTTGGGTGGT ANAATGTNAA TTAANGATTC TTGCAACTGT TTGTGTNATT
R35464    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
H94519    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
N39798    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          551                                                        600
Bikunin   GGGGCTGTGA   CGGAAACA GCAATAATTA CCTGACCAAG GA GGAGTGC
N57374    GGGGCTNTTA AACGGAAANA .CAATAATNA CCTGACCAAA GAAGNAAT..
R35464    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H94519    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
N39798    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H87300    GATTCGGCAC AGGGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTNC
R74593    .......... .......... GCAATAATTA CCTGACCAAG GA.GGAGTGC
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
           601                                                       650
Bikunin    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R35464     CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H94519     CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
N39798     CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H87300     CTCAAGAAAT GTNCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R74593     CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R31730     .......... .......... .......... .......... ..........
R34701     .......... .......... .......... .......... ..........
H02982     .......... .......... .......... .......... ..........
R32676     .......... .......... .......... .......... ..........
T47439     .......... .......... .......... .......... ..........
R73968     .......... .......... .......... .......... ..........
H39840     .......... .......... .......... .......... ..........
H95233     .......... .......... .......... .......... ..........
H39841     .......... .......... .......... .......... ..........
N30199     .......... .......... .......... .......... ..........
T52966     .......... .......... .......... .......... ..........
N29508     .......... .......... .......... .......... ..........
N26919     .......... .......... .......... .......... ..........
N26910     .......... .......... .......... .......... ..........
H16757     .......... .......... .......... .......... ..........
N27732     .......... .......... .......... .......... ..........

           651                                                       700
Bikunin    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R35464     CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H94519     CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
N39798     CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H87300     CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R74593     CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGT.CTCCC AGAAGGCAGG
R31730     .......... .......... .......... .......... ..........
R34701     .......... .......... .......... .......... ..........
H02982     .......... .......... .......... .......... ..........
R32676     .......... .......... .......... .......... ..........
T47439     .......... .......... .......... .......... ..........
R73968     .......... .......... .......... .......... ..........
H39840     .......... .......... .......... .......... ..........
H95233     .......... .......... .......... .......... ..........
H39841     .......... .......... .......... .......... ..........
N30199     .......... .......... .......... .......... ..........
T52966     .......... .......... .......... .......... ..........
N29508     .......... .......... .......... .......... ..........
N26919     .......... .......... .......... .......... ..........
N26910     .......... .......... .......... .......... ..........
H16757     .......... .......... .......... .......... ..........
N27732     .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

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           701                                                       750
Bikunin    ATTCT GAAG ACCACTCCAG CGATATGTT  CAACTAT  G AAGAATACTG
R35464     ATTCTTGAAG ACCACTTCAG CGATATGTTT CAANTATTGN AAGAATAATT
H94519     ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
N39798     ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
H87300     ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R74593     ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R31730     .......... .......... .......... .......... ..........
R34701     .......... .......... .......... .......... ..........
H02982     .......... .......... .......... .......... ..........
R32676     .......... .......... .......... .......... ..........
T47439     .......... .......... .......... .......... ..........
R73968     .......... .......... .......... .......... ..........
H39840     .......... .......... .......... .......... ..........
H95233     .......... .......... .......... .......... ..........
H39841     .......... .......... .......... .......... ..........
N30199     .......... .......... .......... .......... ..........
T52966     .......... .......... .......... .......... ..........
N29508     .......... .......... .......... .......... ..........
N26919     .......... .......... .......... .......... ..........
N26910     .......... .......... .......... .......... ..........
H16757     .......... .......... .......... .......... ..........
N27732     .......... .......... .......... .......... ..........

           751
Bikunin     CACCGCCAA CGCAGT CAC TGGGCC TTG CCGTG CAT  CCTT CCCAC
R35464     GCACCGNCAA CGNATT
H94519     GCACCGCCAA CGCATT.CAC TGGGCC..TG C.GTG.CAT. CCTT.CCCAC
N39798     .CACCGCCAA CGCAGT.CAC TGGGGCCTTG C.GTGGAAT. CCTTTCCCAC
H87300     .CACCGCCAA CGCAGTNCAC TGGGCC.TTG C.GTGGCATN CCTT.CCCAC
R74593     .CACCGCCAA CGCAGT.CAC TGGGCC.TTG CCGTG.CAT. CCTT.CCCAC
R31730     .......... .......... .......... .......... ..........
R34701     .......... .......... .......... .......... ..........
H02982     .......... .......... .......... .......... ..........
R32676     .......... .......... .......... .......... ..........
T47439     .......... .......... .......... .......... ..........
R73968     .......... .......... .......... .......... ..........
H39840     .......... .......... .......... .......... ..........
H95233     .......... .......... .......... .......... ..........
H39841     .......... .......... .......... .......... ..........
N30199     .......... .......... .......... .......... ..........
T52966     .......... .......... .......... .......... ..........
N29508     .......... .......... .......... .......... ..........
N26919     .......... .......... .......... .......... ..........
N26910     .......... .......... .......... .......... ..........
H16757     .......... .......... .......... .......... ..........
N27732     .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

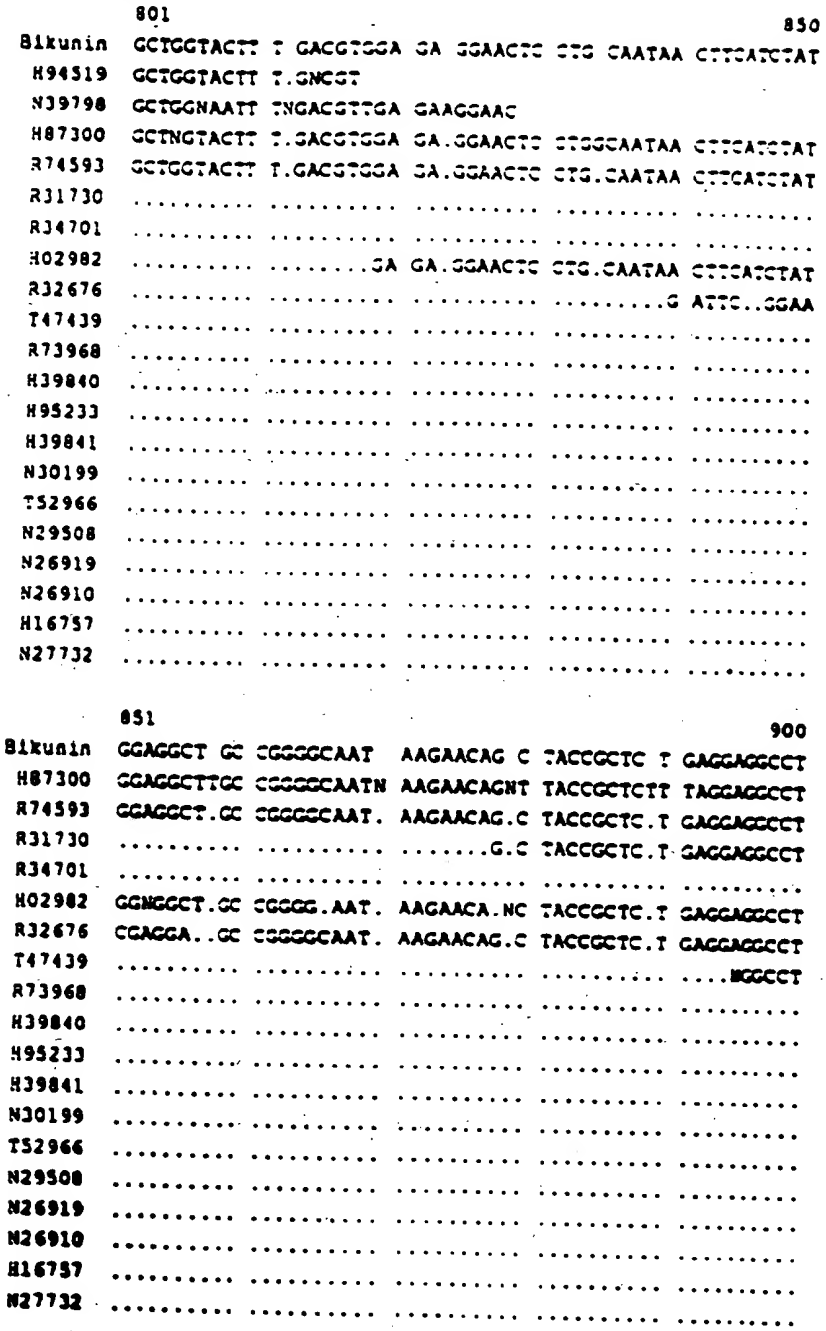

Figure 4C (Con't)

```
          901                                                        950
Bikunin   GCA TGCTC CGCTGCTTCC GC                                    CA GCAGGA
H87300    .GCA.T.... .
R74593    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R31730    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R34701    ..... .....TTCC GC..... ..... .CAAGCAGGA
H02982    .GCG.TGCTC CGCTGCTTCC GCTGTGTGTT CTCTTCCAGG CCA.GCAGGA
R32676    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
T47439    TGCAGTGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R73968    ..... ..... ..... ..... .....
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....

          951                                                        1000
Bikunin   GAA TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC  TGG CGGGGC
R74593    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGGCGGGGC
R31730    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R34701    AAANTCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTCC TGG.CGGGGC
H02982    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R32676    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
T47439    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R73968    ..... ..... ..... ..... ....CGGGGC
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....
```

Figure 4C (Con't)

```
          1001                                                      1050
Bikunin   TGTT CGTGA TGGTGTTGAT CC T CTTCC TGGG AGCCT CC ATGGTC
R74593    TGTTTCGTGA TGGTGTTGAT CCTT..TTCC TGGGGAGCNT CC.ATGGTCT
R31730    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGGGAGCCT CC.ATGGTC.
R34701    TGTT.CGTGA TGGTGTTGAT CCCTCCTTCC CGGG.AGCCT CCCATCGTCC
H02982    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTN.
R32676    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
T47439    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
R73968    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1051                                                      1100
Bikunin   TACC TGAT  CCGGGTGGCA CGGAGG AAC C AGG AGCG TGCCCTGCGC
R74593    TAC..TGATT CCGGGTGGCA AGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
R31730    TACC.TGAT. CCGGGTGGCA CGGAGGGAAC C.AGGGAGCG TGCCCTGCGC
R34701    TACCCTGAT. CCGGGTGGCA CGGAGG.AAC CCAGG.ANCG TGCCCTGCGC
H02982    TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGN
R32676    TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGC
T47439    TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
R73968    TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
H39840    .......... .......... ...GGG.AAC C.AGG.AGCG TGCCCTGCGC
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... ..GAGGAACC C.ANG.AGCT TCCCCTGCGC
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          1201                                                  1250
Bikunin   GGGAGGGG AGACTAT G  TGT GA GCT TTTTTT  AA A TAGA  GG
R31730    .GGGAGGGGG A
R34701    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TA
H02982    GGGGAGGGG. AGATTAT.G. TGTTGA.GTT TTTTTT..AA ANTAG
R32676    GGGGAGGGGG AGANTATTGT TGTTGA.GNT TTTTTTTAAA ATTAGGAGGG
T47439    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
R73968    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
H39840    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
H95233    .......... .......... .......... .......... ..........
H39841    .GGGAGGGGA AAACNAT.G. TGT.GAACCT TTTTTT.AAA A.TAGA..GG
N30199    .GGGAGGNG. AGACTAT.G. TGT.AA.GCT TTTTTT..AA A.TAGA..GG
T52966    .......... .......... .......... .......... ..........
N29508    .GGGAGGGG. AGACTA..G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1251                                                  1300
Bikunin   GATTGACTC  GGATTTG A GT GATC A  TTAGGG  CT GAGGTCTGTT
R32676    GNTTGANTTC GGGNTTTTNA GTTGATCCAT TTAGGGGGNT GAG
T47439    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTNTT
R73968    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
H39840    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
H95233    .......... .......... ........A. TTAGGG..CT GAGGTCTGTT
H39841    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
N30199    GATTGACTC. .GGATTTGGA GT.GATC.A. TTAGGG..CT GAGGTCTGTT
T52966    .......... .......... .......... .......... ..........
N29508    GATTGACTC. .GGATTTG.A GT.GATCNA. TTAGGG..CT GAGGTCTGTT
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1301                                                  1350
Bikunin   TCTCTGGGAG GTAGGACGGC TGCTTCC TG G TC TGGCA  GGGATGGG
T47439    TCTCTNGGAG GTAGGACGA
R73968    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG GGTCTTGGCA .GGGATGGGG
H39840    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
H95233    NCTCTGGGAG NTAGGACGGC TGCTTTCCTG G.TC.TGGCA .GGGATGGG.
H39841    TCNCTGGGAG GTAGGACGGC TGCTCCCCTG G.TC.TGGCA .GGGATGGG.
N30199    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
T52966    .......... .......... .......... ..TC.TGGCA .GGGATGGG.
N29508    TCTCTGGGAG GTAGGACGGC TGCTTCA.TG G.TC.TGGCA .GGGATGGG.
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .........G G.TC.TGGCA .GGGATGGG.
N27732    .......... .......... .....CCCTG GGTCCTGNCA AGGNATGGGG
```

Figure 4C (Con't)

```
          1351                                                    1400
Bikunin   TTTG CTTTG G AAATCCTC T AGGAGGCT CCTCCT CGC ATGG CC TG
R73968    TTTG.CTTTG GGAAATCCTC TTNGGAGGCT CCTCCTTCGC ATGGGCCTTG
H39840    TTTG.CTTTG GAGAATCCTC T.ANGAGGCT CCTCCT.CGC ATGG.CC.TG
H95233    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H39841    TTTG.CTTTG G.AAANCCNC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N30199    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCTTCGC ATGG.CC.TG
T52966    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N29508    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N26919    ......... ......... ....GAGGCT CCTCCT.CGC ATGG.CC.TG
N26910    .....CTTTT GNAAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H16757    TTTGCCTTTG G.AAANCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N27732    TTTG.CTTTG G.AAATCCTC TTAGGAGGCT CCTCCT.CGC ATGG.CC.TG

          1401                                                    1450
Bikunin   CAGT CT GG CAGCAG CCC CGAGTTGTTT  CC TCGCTG ATC GATTTC
R73968    CAGT.CTNGG CAGCANCCCC CGAGTTTTTT TCCTTCGCTG ATCCGATTTC
H39840    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H95233    CAGTTCT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H39841    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTN .CC.TCGCTG ATC.GATNTC
N30199    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
T52966    CAGT.CT.GG CAGCAG..CC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N29508    CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N26919    CAGT.CTTGG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC
N26910    CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATCGGATTTC
H16757    CAGTNCT.GG CAGCAGACCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N27732    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC

          1451                                                    1500
Bikunin   TTT CCTCCA GGTAG  AGT TTTC TTTG  CTTATGTTGA ATTCCATTGC
R73968    TTTTCCTCCA GGTAAGAATT TTTCTTTT
H39840    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H95233    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H39841    TTT.CCCCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ANTCCATTGC
N30199    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
T52966    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N29508    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26919    TTT.CCNCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26910    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H16757    TTTACCCCCA GGTAG..AGT TTTCCTTTGN CTTATGTTGA ATTCCATTGC
N27732    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
```

Figure 4C (Con't)

```
          1501                                                    1550
Bikunin   CTCTTTT CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT GT
H39840    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTTTGT
H95233    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
H39841    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N30199    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
T52966    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N29508    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N26919    CTCTTTT.CN CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N26910    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
H16757    CTCTTTTACT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N27732    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT

          1551                                                    1600
Bikunin   CTGATTTATG G  TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H39840    CTGATTTATG GGTTTTTTTT AAGTAT
H95233    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H39841    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N30199    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
T52966    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N29508    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N26919    CTGATTTATG G..TTTTTTT AAGTNTAAAC AAAAGTTTTT TATTAGCATT
N26910    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H16757    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N27732    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT

          1601                                                    1650
Bikunin   CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
H95233    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAA
H39841    CTGAAAGAAG GAAAGTAAAN TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N30199    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
T52966    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N29508    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N26919    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N26910    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
H16757    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N27732    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC

          1651                                          1689
Bikunin   CTTTAG AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
H39841    CTTTAA.
N30199    CTTTAG.AAT AAA
T52966    CTTTAGGAAT NAAAANAAAA AAGGGTG
N29508    CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AA
N26919    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA A
N26910    CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AAAAAA
H16757    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAA
N27732    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

FIGURE 4D

```
EST consens MLRAEADGVS RLLGSLLLSG VLAADRERSI HDFCLVSKVV GRCRASMPRW  50
EST consens WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATVTEN ATGDLATSRN 100
EST consens AADSSVPSAP RRQDSEDHSS DMFNYEEYCT ANAVTGPCRA SFPRWYFDVE 150
EST consens RNSCNNFIYG GCRGNKNSYR SEEACMLRCF RQQENPPLPL GSKVVVLAGL 200
EST consens FVMVLILFLG ASMVYLIRVA RRNQERALRT VWSSGDDKEQ LVKNTYVL   248
```

FIGURE 4E

```
cDNA
translation                                                    ACC   3
                                                               T    -47

cDNA        TGATCGCGAG ACCCCAACGG CTGGTGGCGT CGCCTGCGCG TCTCGGCTGA  53
translation  .  S  R  D   P  N  G   W  W  R   R  L  R  V   S  A  E  -30

cDNA        GCTGGCCATG GCGCAGCTGT GCGGGCTGAG GCGGAGCCGG GCGTTTCTCG 103
translation  L  A  M   A  Q  L  C   G  L  R   R  S  R   A  F  L  A-13

cDNA        CCCTGCTGGG ATCGCTGCTC CTCTCTGGGG TCCTGGCGGC CGACCGAGAA 153
translation  L  L  G   S  L  L  L   S  G  V   L  A  A   D  R  E    4

cDNA        CGCAGCATCC ACGACTTCTG CCTGGTGTCG AAGGTGGTGG GCAGATGCCG 203
translation R  S  I  H   D  F  C   L  V  S   K  V  V  G   R  C  R   21

cDNA        GGCCTCCATG CCTAGGTGGT GGTACAATGT CACTGACGGA TCCTGCCAGC 253
translation  A  S  M   P  R  W  W   Y  N  V   T  D  G   S  C  Q  L   38

cDNA        TGTTTGTGTA TGGGGGCTGT GACGGAAACA GCAATAATTA CCTGACCAAG 303
translation  F  V  Y   G  G  C   D  G  N  S   N  N  Y   L  T  K    54

cDNA        GAGGAGTGCC TCAAGAAATG TGCCACTGTC ACAGAGAATG CCACGGGTGA 353
translation E  E  C  L   K  K  C   A  T  V   T  E  N  A   T  G  D   71

cDNA        CCTGGCCACC AGCAGGAATG CAGCGGATTC CTCTGTCCCA AGTGCTCCCA 403
translation  L  A  T   S  R  N  A   A  D  S   S  V  P   S  A  P  R   88

cDNA        GAAGGCAGGA TTCTGAAGAC CACTCCAGCG ATATGTTCAA CTATGAAGAA 453
translation   R  Q  D   S  E  D   H  S  S  D   M  F  N   Y  E  E   104

cDNA        TACTGCACCG CCAACGCAGT CACTGGGCCT TGCCGTGCAT CCTTCCCACG 503
translation Y  C  T  A   N  A  V   T  G  P   C  R  A  S   F  P  R  121

cDNA        CTGGTACTTT GACGTGGAGA GGAACTCCTG CAATAACTTC ATCTATGGAG 553
translation  W  Y  F   D  V  E  R   N  S  C   N  N  F   I  Y  G  G 138

cDNA        GCTGCCGGGG CAATAAGAAC AGCTACCGCT CTGAGGAGGC CTGCATGCTC 603
translation   C  R  G   N  K  N   S  Y  R  S   E  E  A   C  M  L   154

cDNA        CGCTGCTTCC GCCAGCAGGA GAATCCTCCC CTGCCCCTTG GCTCAAAGGT 653
translation R  C  F  R   Q  Q  E   N  P  P   L  P  L  G   S  K  V  171

cDNA        GGTGGTTCTG GCGGGGCTGT TCGTGATGGT GTTGATCCTC TTCCTGGGAG 703
translation V  V  L  A   G  L  F   V  M  V   L  I  L  F   L  G  A  188

cDNA        CCTCCATGGT CTACCTGATC CGGGTGGCAC GGAGGAACCA GGAGCGTGCC 753
translation  S  M  V  Y   L  I   R  V  A  R   R  N  Q   E  R  A   204

cDNA        CTGCGCACCG TCTGGAGCTT CGGAGATGA                        782
translation L  R  T  V   W  S  F   G  D                           213
```

FIGURE 4F

```
cDNA          GCACGAGTTG GGAGGTGTAG CGCGGCTCTG AACGCGCTGA GGGCCGTTGA  50
cDNA          GTGTCGCAGG CGGCGAGGGC GCGAGTGAGG AGCAGACCCA GGCATCGCGC 100
cDNA          GCCGAGAAGG CCGGGCGTCC CCACACTGAA GGTCCGGAAA GGCGACTTCC 150
cDNA          GGGGGCTTTG GCACCTGGCG GACCCTCCCG GAGCGTCGGC ACCTGAACGC 200
cDNA          GAGGCGCTCC ATTGCGCGTG CGCGTTGAGG GGCTTCCCGC ACCTGATCGC 250
cDNA          GAGACCCCAA CGGCTGGTGG CGTCGCCTGC GCGTCTCGGC TGAGCTGGCC 300
cDNA          ATGGCGCAGC TGTGCGGGCT GAGGCGGAGC CGGGCGTTTC TCGCCCTGCT 350
translation   M  A  Q  L   C  G  L   R  R  S   R  A  F  L   A  L  L -11

cDNA          GGGATCGCTG CTCCTCTCTG GGGTCCTGGC GGCCGACCGA GAACGCAGCA 400
translation   G  S  L   L  L  S  G   V  L  A   A  D  R   E  R  S  I   7

cDNA          TCCACGACTT CTGCCTGGTG TCGAAGGTGG TGGGCAGATG CCGGGCCTCC 450
translation     H  D  F   C  L  V   S  K  V  V   G  R  C   R  A  S   23

cDNA          ATGCCTAGGT GGTGGTACAA TGTCACTGAC GGATCCTGCC AGCTGTTTGT 500
translation   M  P  R  W   W  Y  N   V  T  D   G  S  C  Q   L  F  V   40

cDNA          GTATGGGGGC TGTGACGGAA ACAGCAATAA TTACCTGACC AAGGAGGAGT 550
translation   Y  G  G   C  D  G  N   S  N  N   Y  L  T   K  E  E  C   57

cDNA          GCCTCAAGAA ATGTGCCACT GTCACAGAGA ATGCCACGGG TGACCTGGCC 600
translation     L  K  K   C  A  T   V  T  E  N   A  T  G   D  L  A   73

cDNA          ACCAGCAGGA ATGCAGCGGA TTCCTCTGTC CCAAGTGCTC CCAGAAGGCA 650
translation   T  S  R  N   A  A  D   S  S  V   P  S  A  P   R  R  Q   90

cDNA          GGATTCTGAA GACCACTCCA GCGATATGTT CAACTATGAA GAATACTGCA 700
translation   D  S  E   D  H  S  S   D  M  F   N  Y  E   E  Y  C  T 107

cDNA          CCGCCAACGC AGTCACTGGG CCTTGCCGTG CATCCTTCCC ACGCTGGTAC 750
translation     A  N  A   V  T  G   P  C  R  A   S  F  P   R  W  Y  123

cDNA          TTTGACGTGG AGAGGAACTC CTGCAATAAC TTCATCTATG GAGGCTGCCG 800
translation   F  D  V  E   R  N  S   C  N  N   F  I  Y  G   G  C  R 140

cDNA          GGGCAATAAG AACAGCTACC GCTCTGAGGA GGCCTGCATG CTCCGCTGCT 850
translation   G  N  K   N  S  Y  R   S  E  E   A  C  M   L  R  C  F 157

cDNA          TCCGCCAGCA GGAGAATCCT CCCCTGCCCC TTGGCTCAAA GGTGGTGGTT 900
translation     R  Q  Q   E  N  P   P  L  P  L   G  S  K   V  V  V  173

cDNA          CTGGCGGGGC TGTTCGTGAT GGTGTTGATC CTCTTCCTGG GAGCCTCCAT 950
translation   L  A  G  L   F  V  M   V  L  I   L  F  L  G   A  S  M 190

cDNA          GGTCTACCTG ATCCGGGTGG CACGGAGGAA CCAGGAGCGT GCCCTGCGCA 1000
translation   V  Y  L  I   R  V  A   R  R  N   Q  E  R   A  L  R  T 207

cDNA          CCGTCTGGAG CTCCGGAGAT GACAAGGAGC AGCTGGTGAA GAACACATAT 1050
translation     V  W  S   S  G  D   D  K  E  Q   L  V  K   N  T  Y  223

cDNA          GTCCTGTGAC CGCCCTGTCG CCAAGAGGAC TGGGGAAGGG AGGGGAGACT 1100
translation   V  L  *                                                225
```

FIGURE 4F (Con't)

```
cDNA        ATGTGTGAGC TTTTTTTAAA TAGAGGGATT GACTCGGATT TGAGTGATCA 1150
cDNA        TTAGGGCTGA GGTCTGTTTC TCTGGGAGGT AGGACGGCTG CTTCCTGGTC 1200
cDNA        TGGCAGGGAT GGGTTTGCTT TGGAAATCCT CTAGGAGGCT CCTCCTCGCA 1250
cDNA        TGGCCTGCAG TCTGGCAGCA GCCCCGAGTT GTTTCCTCGC TGATCGATTT 1300
cDNA        CTTTCCTCCA GGTAGAGTTT TCTTTGCTTA TGTTGAATTC CATTGCCTCC 1350
cDNA        TTTTCTCNAT CACAGAAGTG ATGTTGGAAT CGTTTCTTTT GTTTGTCTGA 1400
cDNA        TTTATGGTTT TTTTAAGTAT AAACAAAAGT TTTTTATTAG CATTCTGAAA 1450
cDNA        GAAGGAAAGT AAAATGTACA AGTTTAATAA AAAGGGGCCT TCCCCTTTAG 1500
cDNA        AATAAATTTC CAGCATGTTG CTTTCAAAAA AAAAAAAAAA AAAA
1550
```

FIGURE 4G

```
EST cons ns                                                    MLR AEADGVSRLL GSLLLSGVLA  -1
PCR cl ne                                                   MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
λcDNA clone                                                 MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

EST consens ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
PCR clone   ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
λcDNA clone ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

EST consens YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
PCR clone   YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
λcDNA clone YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

EST consens NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
PCR clone   NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
λcDNA clone NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

EST consens ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
PCR clone   ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
λcDNA clone ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

EST consens QERALRTVWS SGDDKEQLVK NTYVL                          225
PCR clone   QERALRTVWS FGD                                      213
λcDNA clone QERALRTVWS SGDDKEQLVK NTYVL                          225
```

FIGURE 4G

```
EST consens                                          MLR AEADGVSRLL GSLLLSGVLA  -1
PCR clone                                          MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
λcDNA clone                                        MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

EST consens ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
PCR clone   ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
λcDNA clone ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

EST consens YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
PCR clone   YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
λcDNA clone YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

EST consens NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
PCR clone   NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
λcDNA clone NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

EST consens ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
PCR clone   ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
λcDNA clone ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

EST consens QERALRTVWS SGDDKEQLVK NTYVL                            225
PCR clone   QERALRTVWS FGD                                        213
λcDNA clone QERALRTVWS SGDDKEQLVK NTYVL                            225
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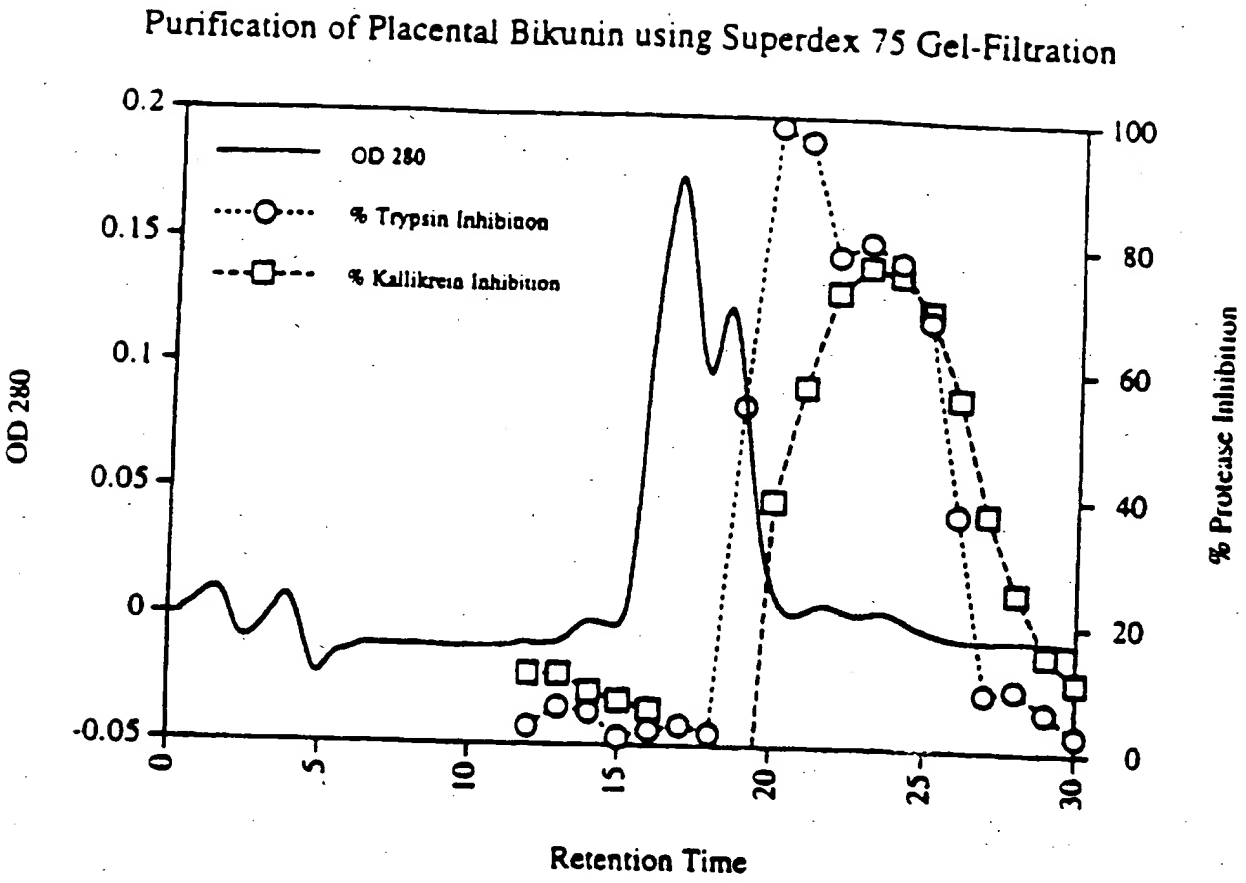

Purification of Placental Bikunin using Superdex 75 Gel-Filtration
OD 280
% Trypsin Inhibition
% Kallikrein Inhibition
OD 280
% Protease Inhibition
Retention Time
0.2
0.15
0.1
0.05
0
-0.05
100
80
60
40
20
0
0
5
10
15
20
25
30

FIGURE 5

FIGURE 6

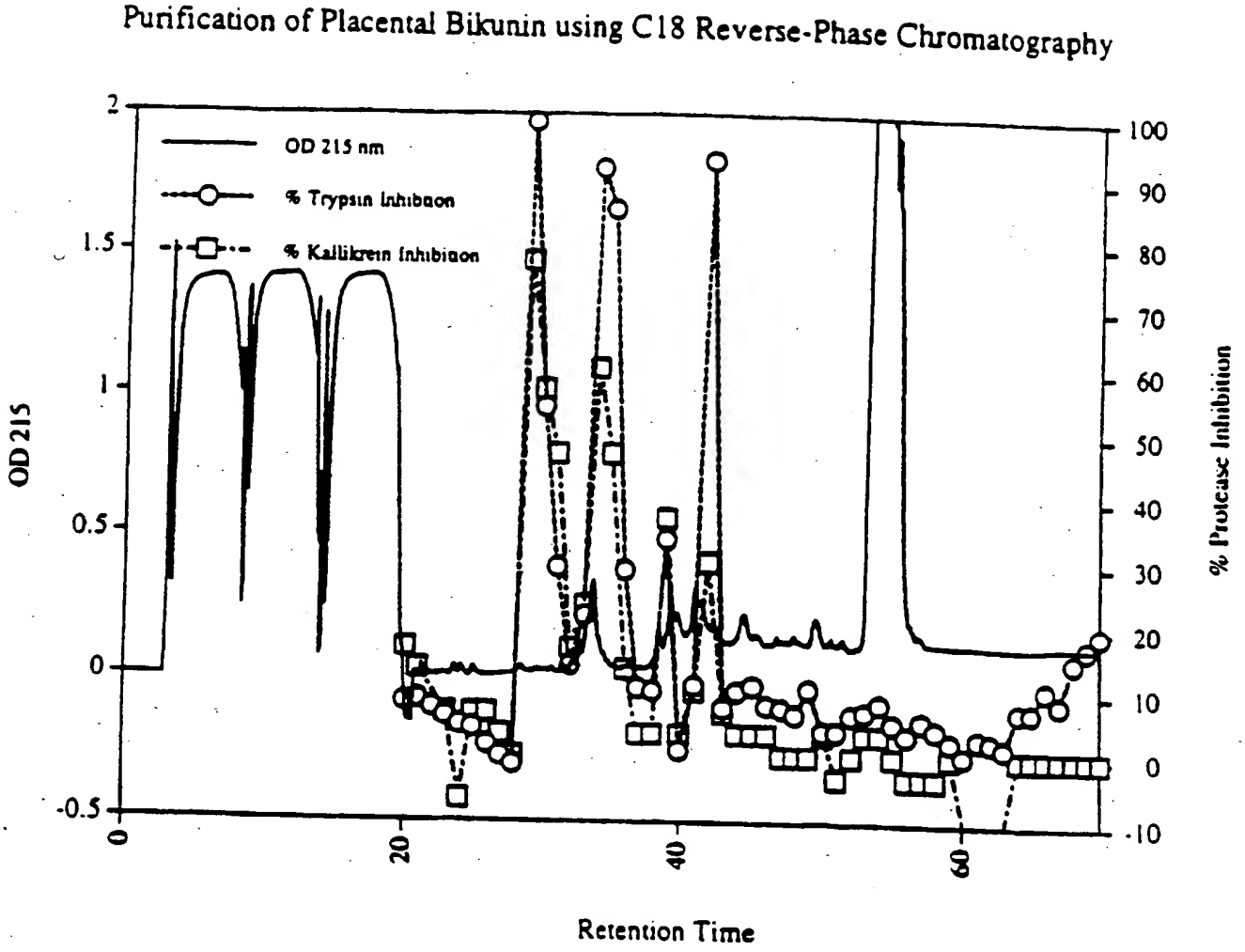

Purification of Placental Bikunin using C18 Reverse-Phase Chromatography
OD 215 nm
% Trypsin Inhibition
% Kallikrein Inhibition
OD 215
% Protease Inhibition
Retention Time
2
1.5
1
0.5
0
-0.5
100
90
80
70
60
50
40
30
20
10
0
-10
0
20
40
60

Figure 7

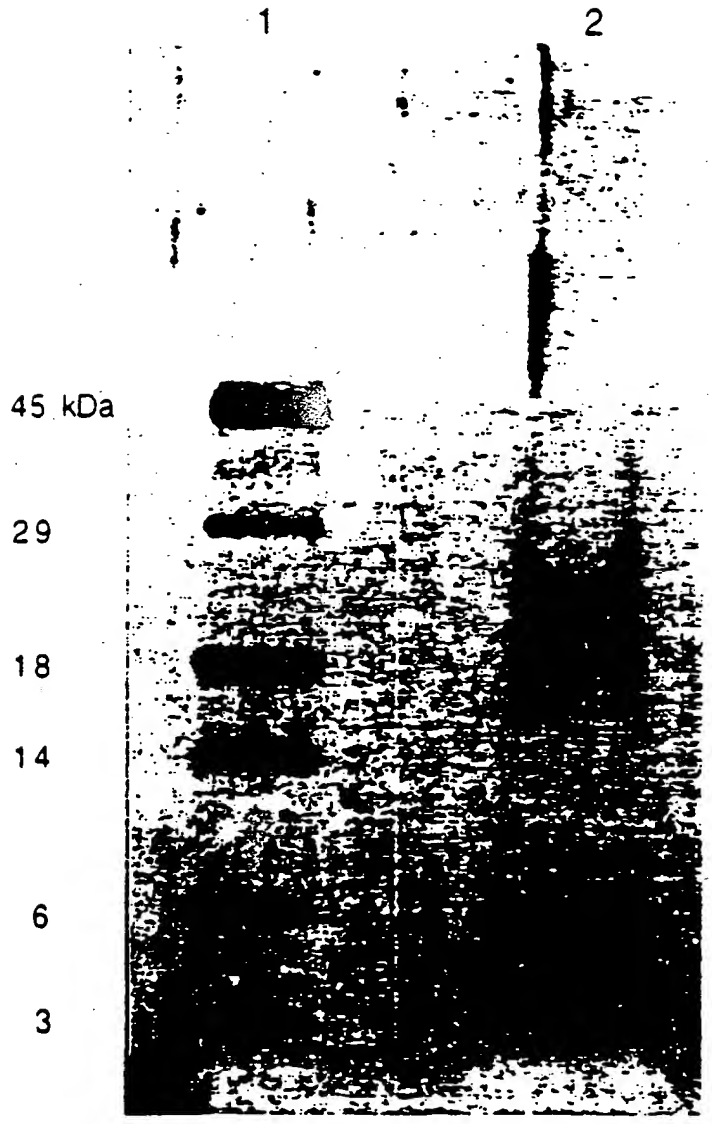


Figure 8A

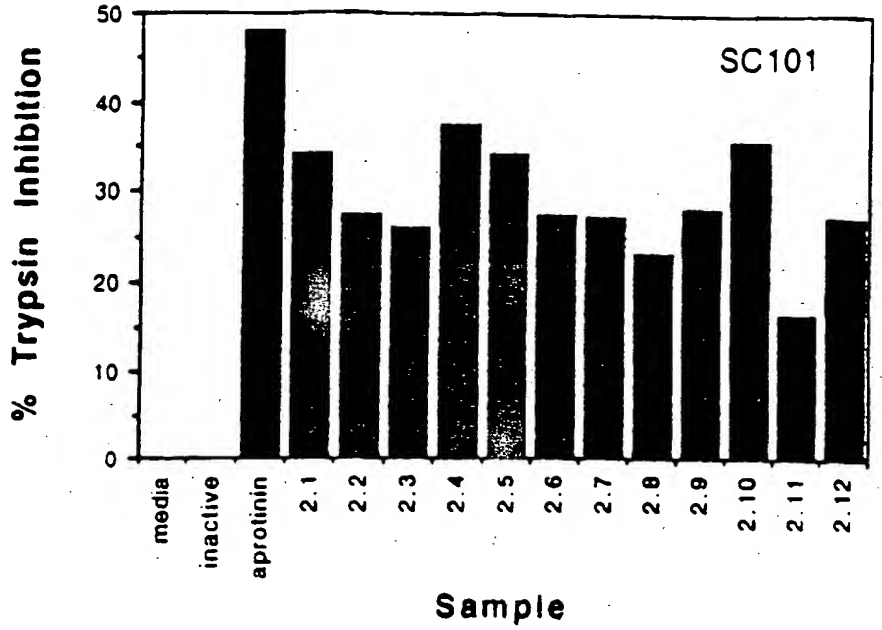


Figure 8B

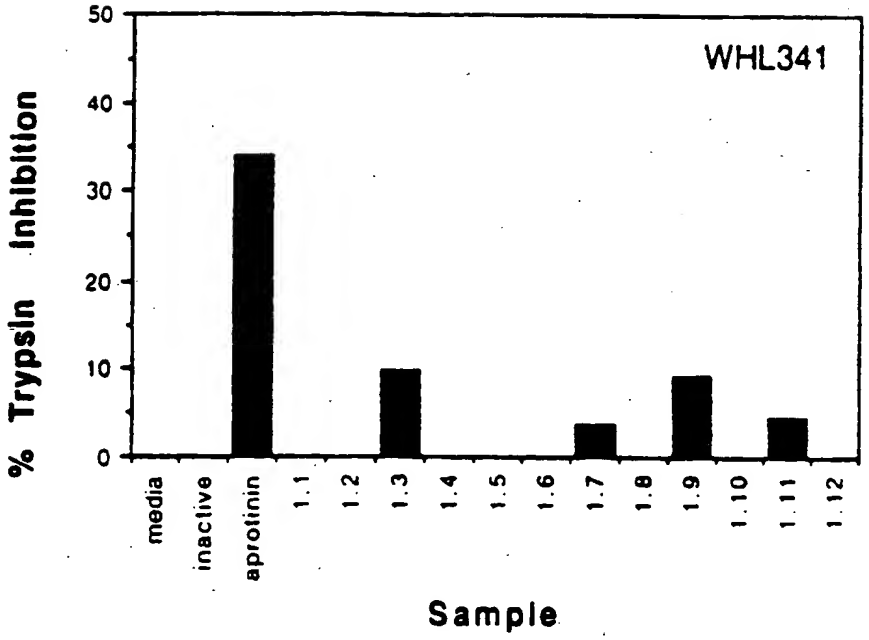


Figure 9

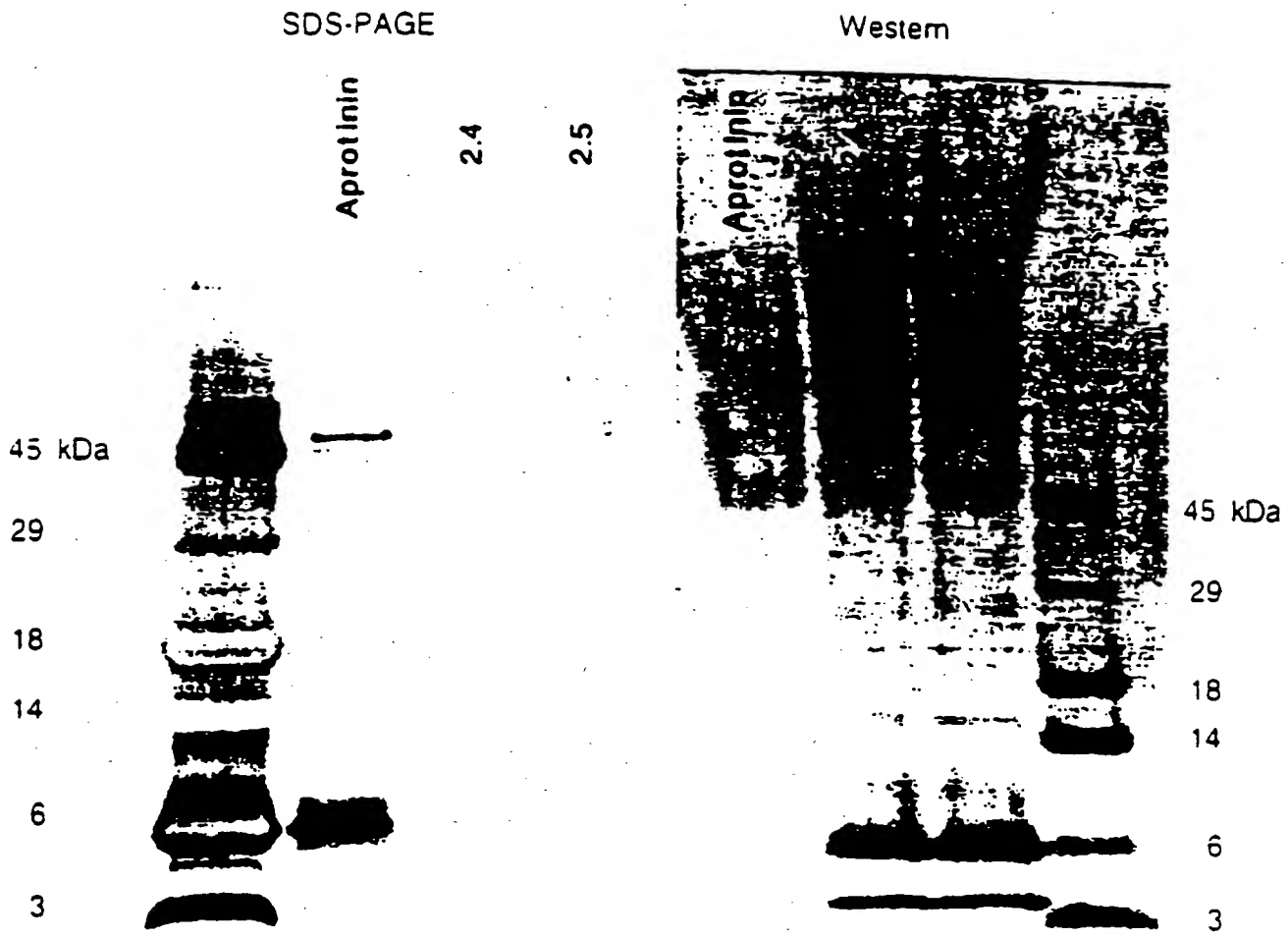

SDS-PAGE
Western
AprotInIn
2.4
2.5
AprotInIa
45 kDa
29
18
14
6
3
45 kDa
29
18
14
6
3

Figure 10

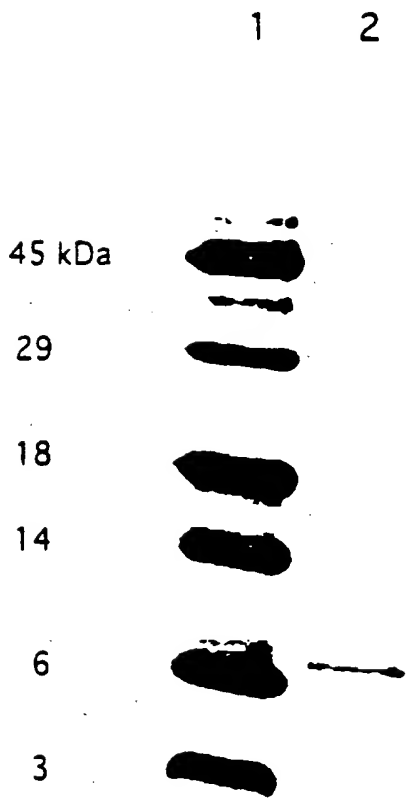


Figure 11

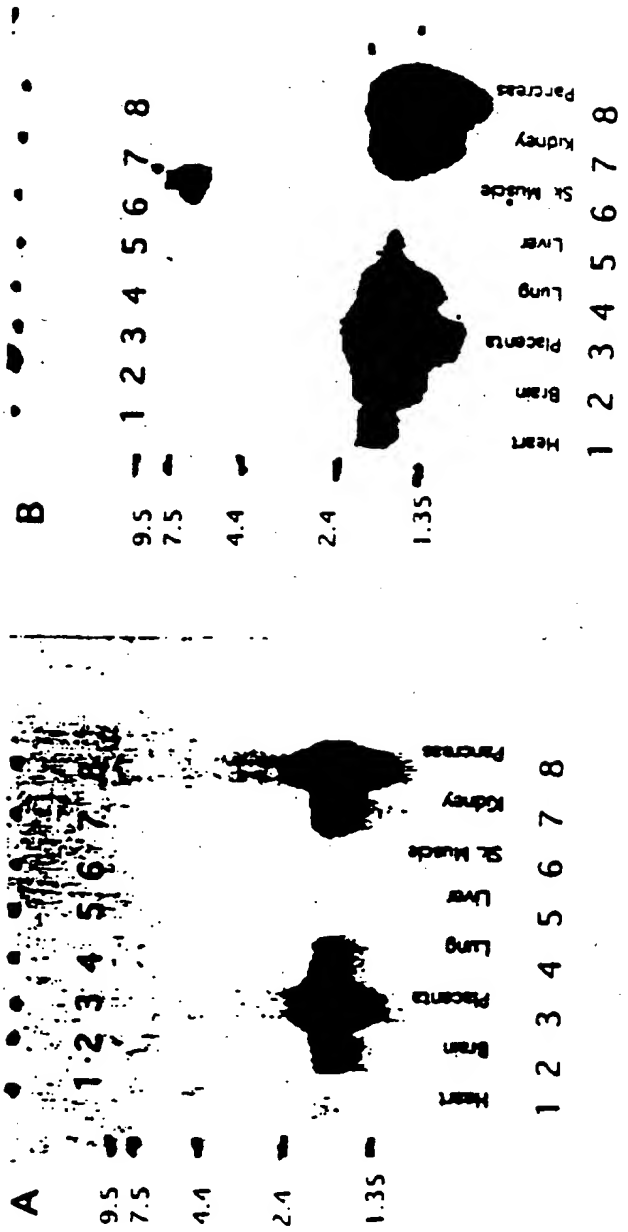

A
B
9.5
7.5
4.4
2.4
1.35
1 2 3 4 5 6 7 8
Heart
Brain
Placenta
Lung
Liver
Sk. Muscle
Kidney
Pancreas

Figure 12

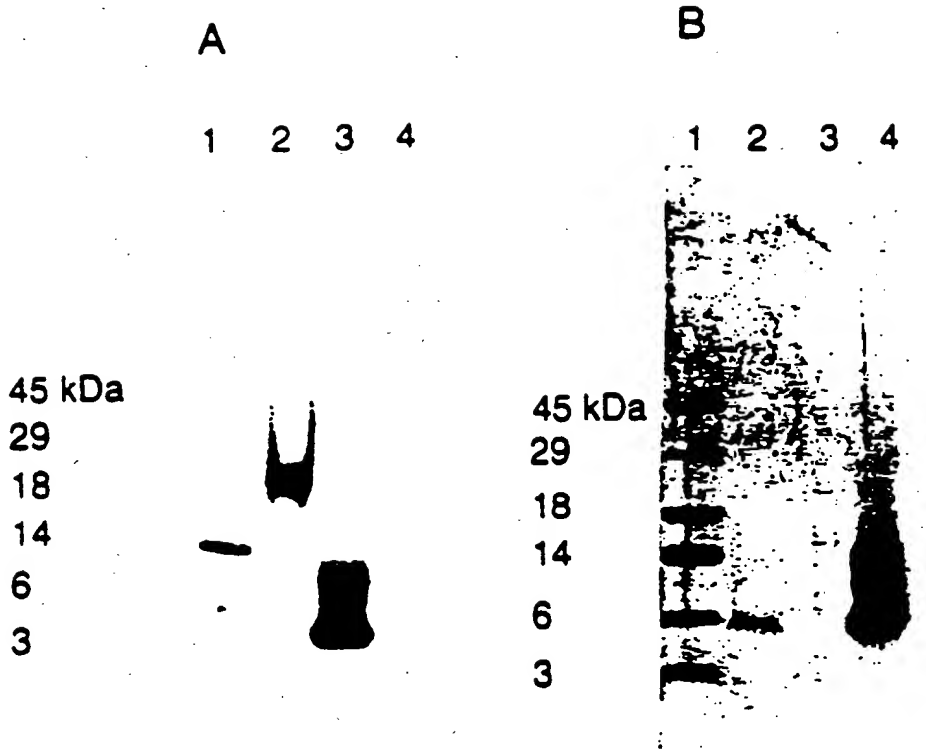


Figure 13

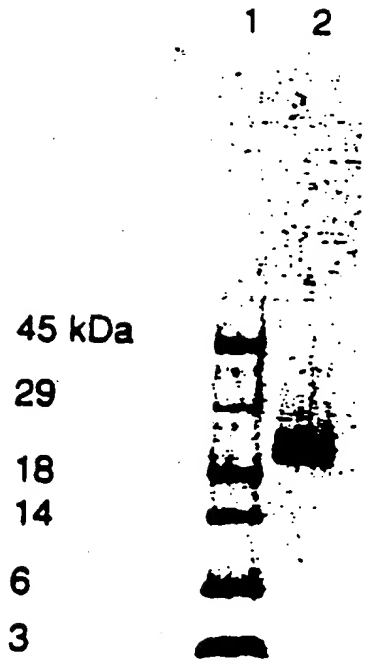
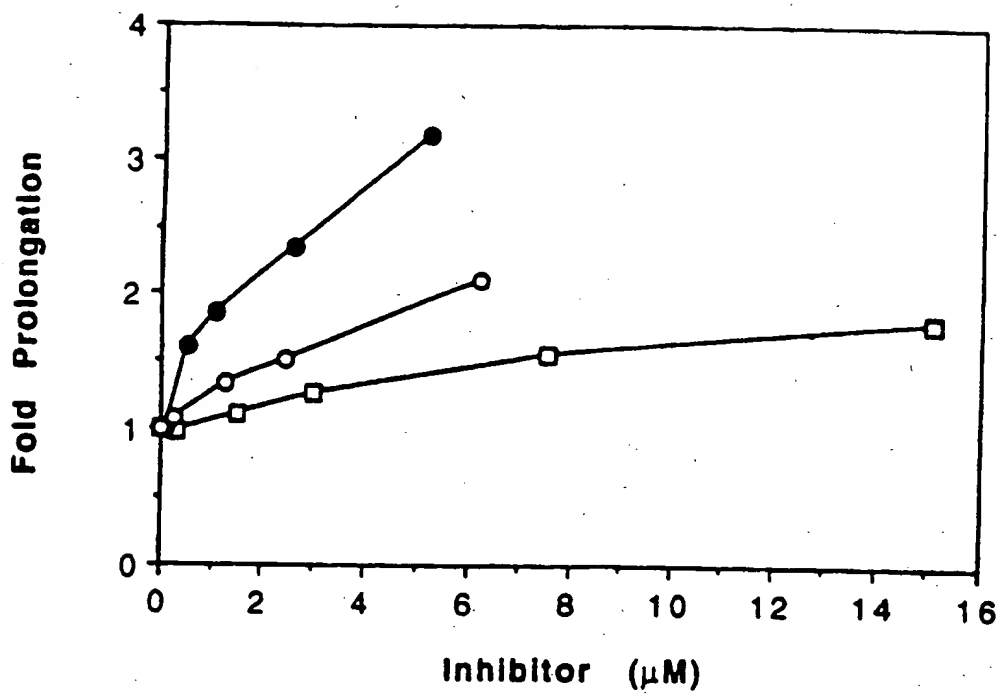


Figure 14



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/15, C07K 14/81, A61K 38/57 — A3

(11) International Publication Number: WO 97/33996

(43) International Publication Date: 18 September 1997 (18.09.97)

(21) International Application Number: PCT/US97/03894

(22) International Filing Date: 10 March 1997 (10.03.97)

(30) Priority Data:

60/013,106	11 March 1996 (11.03.96)	US
60/019,793	14 June 1996 (14.06.96)	US
08/725,251	4 October 1996 (04.10.96)	US

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Filed on	11 March 1996 (11.03.96)

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: TAMBURINI, Paul, P. [GB/US]; 36 Misty Mountain Road, Kensington, CT 06460 (US). DAVIS, Gary [US/US]; 137 Tanglewood Circle, Milford, CT 06460 (US). DELARIA, Katherine, A. [US/US]; 180 West Walk Street, West Haven, CT 06516 (US). MARLOR, Christopher, W. [US/US]; 11 Robertson Drive, Bethany, CT 06524 (US). MULLER, Daniel, K. [US/US]; 253 Hemlock Hill Road, Orange, CT 06477 (US).

(74) Agents: CHAO, Mark et al.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 13 November 1997 (13.11.97)

(54) Title: HUMAN BIKUNIN

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 97/03894

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/81 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R35464 Sequence reference HS46499, May 4, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039653 see the whole document ---	1-6,11
X	EMBL/GENBANK DATABASES Accession no N39798 Sequence reference HS798277, January 26, 1996 L. HILLIER ET AL: "The WasU-Merck EST Project" XP002039654 see the whole document --- -/--	1-6,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 1997	01. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,	Van der Schaal, C

INTERNATIONAL SEARCH REPORT

Interm al Application No
PCT/US 97/03894

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R74593 Sequence reference HS593137,June 9, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039655 see the whole document	1-6,11
P,X	EP 0 758 682 A (MITSUBISHI CHEM CORP) 19 February 1997 see the whole document	1-11
P,A	JOURNAL OF BIOLOGICAL CHEMISTRY 272 (10). 1997. 6370-6376. ISSN: 0021-9258, XP002039700 SHIMOMURA T ET AL: "Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor."	
A	DATABASE MEDLINE accession no 94289695, 1 July 1994 J. WOJTA ET AL: "Hepatocyte growth factor stimulates expression of plasminogen activator inhibitor type 1 and tissue factor in HepG2 cells" XP002039702 see abstract & BLOOD, vol. 84, no. 1, 1994, pages 151-157,	7-10
A	JOURNAL OF BIOLOGICAL CHEMISTRY 271 (7). 1996. 3615-3618. ISSN: 0021-9258, XP002039701 MIYAZAWA K ET AL: "Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator."	7-10

INTERNATIONAL SEARCH REPORT

Intr ~ational application No

PCT/US 97/03894

Box I Observations where certain claims were f und unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1. [X] Claims Nos.
because they relate to subject matter not required to be searched by this Authority, namely

Remark: Although claim(s) 7-9
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3. [ ] Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos..

4. [ ] No required additional search fees were timely paid by the applicant Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest

[ ] No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ial Application No
PCT/US 97/03894

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0758682 A	19-02-97	JP 9095498 A	08-04-97

CORRECTED VERSION*

PCT

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BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
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ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan

Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

Protein Serine Protease Inhibitor

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98, 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hemorrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979)Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976) , Artzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al., in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant invention embodies a protein having the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 1)

In a prefered embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK 170
(SEQ ID NO: 52)

In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH2-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

```
AGSFLAWLGSLLLSGVLA                                          -1
ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF         100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE         150
ACMLRCFRQQENPPLPLGSKVVVLAGAVS                               179
```

(SEQ ID NO: 2)

In a prefered embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

```
MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
```

(SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

```
MLR AEADGVSRLL GSLLLSGVLA  -1
```

(SEQ ID NO: 54)

In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH2-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid sequence:

```
IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRCFRQ                                            159
```

(SEQ ID NO: 3)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```
CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRC                                               156
```

(SEQ ID NO: 50).

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATV 64
(SEQ ID NO: 4)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-61)" having the amino acid sequence:

CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKC 61
(SEQ ID NO: 5)

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC 156
(SEQIDNO:7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS 92
(SEQIDNO:8)

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

```
1)EST                                MLR AEADGVSRLL GSLLLSGVLA   -1
2)PCR                              MAQLCGL RRSRAFLALL GSLLLSGVLA, -1
3)λcDNA                            MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

1)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
2)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
3)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

1)YLTKEECLFK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
2)YLTKEECLFK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
3)YLTKEECLFK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

1)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
2)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
3)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

1)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
2)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
3)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

1)QERALRTVWS SGDDKEQLVK NTYVL                             225
2)QERALRTVWS FGD                                         213
3)QERALRTVWS SGDDKEQLVK NTYVL                            225
```

where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO:47, and 3) is lambda cDNA clone SEQ ID NO:49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

```
EST     VVVLAGLFVM VLILFLGASM VYLIRVARRN   200
EST     QERALRTVWS SGDDKEQLVK NTYVL        225
```

(SEQ ID NO: 69)

a transmembrane amino acid sequence:

```
PCR     VVVLAGLFVM VLILFLGASM VYLIRVARRN   200
PCR     QERALRTVWS FGD                     213
```

(SEQ ID NO: 68)

or a transmembrane amino acid sequence:

```
λcDNA   VVVLAGLFVM VLILFLGASM VYLIRVARRN   200
λcDNA   QERALRTVWS SGDDKEQLVK NTYVL        225
```

(SEQ ID NO: 67) .

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In an another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa^{1} through Xaa^{32} in the amino acid sequence for native placental bikunin. Substitutions at Xaa^{1} through Xaa^{16} are also preferred for variants of bikunin (7-64), while substitutions at Xaa^{17} through Xaa^{32} are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

Ala Asp Arg Glu Arg Ser Ile Xaa^{1} Asp Phe 10
Cys Leu Val Ser Lys Val Xaa^{2} Gly Xaa^{3} Cys 20
Xaa^{4} Xaa^{5} Xaa^{6} Xaa^{7} Xaa^{8} Xaa^{9} Trp Trp Tyr Asn 30
Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa^{10} 40
Tyr Xaa^{11} Gly Cys Xaa^{12} Xaa^{13} Xaa^{14} Ser Asn Asn 50
Tyr Xaa^{15} Thr Lys Glu Glu Cys Leu Lys Lys 60
Cys Ala Thr Xaa^{16} Thr Glu Asn Ala Thr Gly 70
Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp 80
Ser Ser Val Pro Ser Ala Pro Arg Arg Gln 90
Asp Ser Glu His Asp Ser Ser Asp Met Phe 100
Asn Tyr Xaa^{17} Glu Tyr Cys Thr Ala Asn Ala 110
Val Xaa^{18} Gly Xaa^{19} Cys Xaa^{20} Xaa^{21} Xaa^{22} Xaa^{23} Xaa^{24} 120
Xaa^{25} Trp Tyr Phe Asp Val Glu Arg Asn Ser 130
Cys Asn Asn Phe Xaa^{26} Tyr Xaa^{27} Gly Cys Xaa^{28} 140
Xaa^{29} Xaa^{30} Lys Asn Ser Tyr Xaa^{31} Ser Glu Glu 150
Ala Cys Met Leu Arg Cys Phe Arg Xaa^{32} Gln 160
Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys 170
Val Val Val Leu Ala Gly Ala Val Ser 179
(SEQ ID NO: 11).

where Xaa^{1} - Xaa^{32} each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa^{1}-Xaa^{32} is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa^1 is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa^1 is His or Pro; or wherein Xaa^2 is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa^2 is Val or Thr; or wherein Xaa^3 is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa^3 is Arg or Pro; or wherein Xaa^4 is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa^4 is Arg or Lys; or wherein Xaa^5 is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa^5 is Ala; or wherein Xaa^6 is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa^6 is Ser or Arg ; or wherein Xaa^7 is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^7 is Met or Ile; or wherein Xaa^8 is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa^8 is Pro or Ile; or wherein Xaa^9 is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^9 is Arg; or wherein Xaa^{10} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa^{10} is Val; or wherein Xaa^{11} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{11} is Gly; or wherein Xaa^{12} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa^{12} is Asp or Arg; or wherein Xaa^{13} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{14} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{15} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{15} is Leu or Lys; or wherein Xaa^{16} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa^{16} is Val or Ala; or wherein Xaa^{17} is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa^{17} is Glu or Pro; or wherein Xaa^{18} is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa^{18} is Thr; or wherein Xaa^{19} is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa^{19} is Pro; or wherein Xaa^{20} is an amino acid residue selected from the group consisting of Arg, Lys, Gln and

Ser, in particular wherein Xaa^{20} is Arg or Lys; or wherein Xaa^{21} is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa^{21} is Ala; or wherein Xaa^{22} is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa^{22} is Ser or Arg ; or wherein Xaa^{23} is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^{23} is Phe or Ile; or wherein Xaa^{24} is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa^{24} is Pro or Ile; or wherein Xaa^{25} is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^{25} is Arg: or wherein Xaa^{26} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa^{26} is Val or Ile; or wherein Xaa^{27} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{27} is Gly; or wherein Xaa^{28} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa^{28} is Arg; or wherein Xaa^{29} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{30} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{31} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{31} is Arg or Lys; or wherein Xaa^{32} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa^{32} is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14),

and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus

oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migration was from top

to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with $CaCl_2$. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses

pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery, or with major trauma.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects , as well as the cost of surgery. The methods are thus useful in reducing blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery , as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40

μg/ml (see Pixley, et al. (1993) *Meth. in Enz.*, 222, 51-64) and is activated by tissue and plasma kallikrein. Once activated, it participates in the intrinsic pathway of blood coagulation which is activated when blood or plasma contacts a "foreign" or anionic surface. Once activated, Factor XIIa can then cleave and activate a number of other plasma proteases including Factor XI, prekallikrein, and C1 of the complement system. Thus Factor XII may be involved in causing hypotensive reactions since activated kallikrein can cleave kininogen releasing bradykinin (see Colman, (1984) *J. Clin. Invest.*, 73, 1249).

Sepsis is a disease that results from bacterial infection due to bacterial endotoxin or lipopolysaccharide (LPS). Exposure of Factor XII to LPS results in the activation of Factor XII. Patients with sepsis frequently have symptoms of intravascular coagulation which may also be due to activation of Factor XII by LPS. Septic shock can result from bacterial infection and is associated with fever, low systemic vascular resistance, and low arterial pressure. It is a common cause of death in intensive care units in the United States, where seventy five percent of the patients that die from septic shock have a persistent hypotension (see Parillo, et al. (1989) *Ann Rev. Med.*, 40, 469-485).

Adult respiratory distress syndrome is characterized by pulmonary edema, hypoxemia, and decreased pulmonary compliance. The pathogenesis of the disease is currently unknown although the proteolytic pathways of coagulation and fibrinolysis are believed to play a role (see Carvalho, et al. (1988) *J. Lab Clin. Med.*, 112: 270-277).

The proteins of the instant invention are also a novel human Kunitz type inhibitor of kallikrein, an activator of Factor XII. Thus another object of the current invention is to present a method for the prophylactic or therapeutic treatment of systemic inflammatory reactions such as septic shock, adult respiratory distress syndrome (ARDS), preeclampsia, multiple organ failure and disseminated intravascular coagulation (DIC). The therapeutic or prophylactic administration of the peptides of the instant invention would result in the modulation of these inflammatory conditions and be beneficial to the patient.

Plasmin plays an important role in extracellular matrix degradation and the activation of matrix-metallo protease (MMP) cascades. Collectively these proteases mediate migration of and tissue invasion by both endothelial cells during angiogenesis/neovascularization, and cancer cells during metastasis. Neovascularization is essential to support tumor growth and metastasis is a process which mediates the spreading of tumors and which is associated with

extremely poor patient prognosis.

Several preclinical studies suggest that Kunitz like serine protease inhibitors with a protease specificity similar to aprotinin are useful as medicaments for cancer. For example, aprotinin reduced tumor growth and invasion, with increased tumor necrosis when administered to hamsters bearing a highly invasive fibrosarcoma or to mice bearing a similarly malignant mammary carcinoma (Latner et al., (1974), Br. J. Cancer 30: 60-67; Latner and Turner, (1976), Br. J. Cancer 33: 535-538). Furthermore, administration of 200,000 KIU of aprotinin i.p. to C57B1/6 Cr male mice on days 1 to 14 post-inoculation with Lewis lung carcinoma cells, reduced pulmonary metastases by 50% although had no effect on primary tumor mass (Giraldi et al., (1977) Eur. J. Cancer, 13: 1321-1323). Similarly, administration of 10,000 KIU i.p. on each of days 13-16 post-inoculation of C57BL/6J mice with Lewis tumor cells inhibited pulmonary metastases by 90% without affecting the primary tumor growth (Uetsuji et al., (1992), Jpn. J. Surg. 22: 429-442). In this same study, administration of plasmin or kallikrein with the same dosing schedule was argued to increase the number of pulmonary metastases. These results prompted the authors to suggest that perioperative administration of aprotinin to cancer patients may reduce the likelihood of metastases. Black and Steger (1976, Eur. J. Pharmacol., 38: 313-319) found that aprotinin inhibited the growth of the transplanted rodent Murphy-Strum lymphosarcoma in rats and suggested that the effect involved the inhibition of the kinin-forming enzyme system. Twice daily i.p. injection of female ddY mice with 10,000 KIU of aprotinin for 7 weeks to mice each bearing a single autochtonous squamous cell carcinoma resulting from 3-methylcholanthrene treatment reduced the growth rate of the primary tumors by 90%. In some animals tumor regression was observed. While all vehicle treated animals had died within the seven weeks, all of the aprotinin treatment group remained alive. Reduced tumor growth was associated with hyperkeratosis (Ohkoshi, Gann (1980), 71: 246-250).

Clinically, a surgically cured group of 26 patients who received aprotinin i.v. exhibited a 70% survival two years post surgery with no recurrence of tumors whereas a placebo group of 26 patients at the same time exhibited only a 38% survival with a significant rate of tumor recurrence (Freeman et al. Br. Soc. Gastroenterol. (1980) supplement A: 902). In a case study (Guthrie et al., Br. J. Clin. Pract (1981) 35: 330-332), administration of bromocriptine plus aprotinin to a patient with advanced cancer of the cervix caused remission. Aprotinin was administerd both as a 500,000 KIU i.p. bolus every eight hours concurrently

with a continuous i.v. infusion of aprotinin at a rate of 200,000 KIU per 6 hr for a total of seven days once a month. Treatment was ended at the end of the fourth month due to the development of an allergic reaction to aprotinin. More recent evidence has further underscored a role of plasmin as a target for these effects of aprotinin on metastases.

The mechanism for these events could be related to the fact that aprotinin blocks the invasive potential of cancer cell lines (Liu G., et al., Int J. Cancer (1995), 60: 501-506). Furthermore, since the proteins of the instant invention are also potent inhibitors of plasmin and kallikrien, they are contemplated for use as anti-cancer agents. For example they are contemplated for use in blocking primary tumor growth by restricting neovascularization, primary tumor invasion and in blocking metastasis through inhibition of tissue infiltration. The compounds may be administered locally to tumors or systemically. In a preferred mode of treatment, the protein would be administered perioperatively during tumor debulking to minimize the risk of metastasis. In such a regime, the blood sparing properties of the compound would be additionally advantageous in providing a clearer surgical field of view. Another preferred mode of administration would be as a combination therapy with either MMP inhibitors or chemotherapy. An additional preferred mode of administration would be as a locally administered gene therapy designed to achieve selective expression of placental bikunin within the tumor cells, or their associated stroma and vascular beds.

Preferred types of cancers targeted for therapy would be vasular-dependent solid tumors such as breast, colon, lung, prostate and ovarian carcinomas which exhibit a high metastatic potential, and those for which local delivery of a high concentration of the protein is feasible such as lung cancers through pulmonary delivery, colon carcinomas through hepatic delivery to liver metastasis, or skin cancers such as head and neck carcinomas or melanomas through subcutaneous delivery. Since the proteins of the present invention are of human origin they would be less likely to be associated with allergic or anaphylactic reactions of the kind observed by Guthrie et al., *supra*, upon reuse.

Additionally, the proteins of the present invention are contemplated for use in the reduction of thromboembolic complications associated with activation of the intrinsic pathway of coagulation. This would include prevention of pulmonary embolism in late stage cancer patients, a frequent cause of death (Donati MB., (1994), Haemostasis 24: 128-131).

Edema of the brain and spinal cord is a complication resulting from traumatic brain or spinal cord injury, stroke, cerebral ischemia, cerebral and sub-arachnoid hemhorrhage, surgery (including open heart surgery), infectious diseases such as encephalitis and meningitis, granulomatous diseases such as Sarcoid and focal or diffuse carcinomas, and is a contributor to the high level of morbidity and death following these events. Bradykinin is known to disrupt the blood brain barrier experimentally (Greenwood J., (1991), Neuroradiology, 33: 95-100; Whittle et al., (1992), Acta Neurochir., 115: 53-59), and infusion of bradykinin into the internal carotid artery induced brain edema in spontaneously hypertensive rats (SHR) subjected to common carotid artery occlusion (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191). Elevated levels of bradykinin are found in extracellular fluids following trauma in a model involving traumatized rat spinal chord (Xu et al., (1991), J. Neurochem, 57: 975-980), and in plasma and tissue from rats with brain edema resulting from cerebral ischaemia (Kamiya et al., (1993), Stroke, 24: 571-575). Bradykinin is released from high molecular weight kininogen by serine proteases including kallikrein (Coleman (1984) J. Clin Invest., 73: 1249), and the serine protease inhibitor aprotinin was found to block the magnitude of brain edema resulting from cerebralschemia in SHR rats (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191; Kamiya et al., (1993), Stroke, 24: 571-575) and rabbits subjected to a cold lesion of the brain (Unterberg et al., (1986), J. Neurosurgery, 64: 269-276).

These observations indicate that brain edema results from local proteolytic release of kinins such as bradykinin from high molecular weight kininogen, followed by bradykinin-induced increases in blood brain barrier permeability. Accordingly, placental bikunin and fragments thereof are contemplated as medicaments for the prevention of edema in patients at risk for this condition, particularly those of high risk of mortality or brain injury. This would include head and spinal trauma patients, polytrauma patients, patients undergoing surgery of the brain or spinal cord and their associated vessels or other generalsurgeries including open-heart surgery, patients who have suffered from a stroke, cerebral or sub-arachnoid hemorrhage, infectious diseases of the brain, granulomatous disease of the brain or diffuse or focal carcinomas and tumors of the brain or any conditions such as multiple sclerosis involving breakdown of the blood brain barrier or patients suffering from any other inflammatory processes of the brain or spinal cord. Patients would receive an administration of placental bikunin either as an infusion or bolus injection, intravenously or intracranially. Additional doses of placental bikunin

could be administered intermittently over the following one to three weeks. Dose levels would be designed to attain circulating concentrations in excess of those required to neutralize elevations in plasma levels or bradykinin and other vasoactive peptides formed through the action of serine proteases, and sufficient to reduce edema. Since the protein is of human origin, repeated administration in this course of therapy would not lead to development of an immune reaction to the protein. Placental bikunin and fragments thereof would be contemplated for monotherapy or prophylacsis as well as for use in combination with other medicaments such as neurotherapeutics and neuroprotectants.

Recent evidence (Dela Cadena R. A. at al., (1995), FASEB J. 9: 446-452) has indicated that the contact activation pathway may contribute to the pathogenesis of arthritis and anemia, and that kallikrein inhibitors may be of therapeutic benefit. Accordingly, protease inhibitors of the present invention are contemplated according to their capacity to inhibit human kallikrein, as medicaments for the treatment of arthritis and anemia in humans.

Treatment of male non-insulin diabetic (NIDDM) patients with aprotinin significantly improved total glucose uptake and decreased the metabolic clearance rate of insulin (Laurenti et al., (1996), Diabetic Medicine 13: 642-645). Accordingly, the human proteins of the present invention are contemplated for chronic use as medicaments for the treatment of NIDDM.

Daily treatment of patients at risk of preterm delivery with urinary trypsin inhibitor for two weeks significantly reduced recurrent uterine contractions (Kanayama et al., (1996), Eur J. Obstet. Gynecol. & Reprod. Biol. 67: 133-138). Accordingly, the human proteins of the present invention are contemplated for use in the prevention of preterm delivery.

Aprotinin has been shown to stimulate differentiation of mouse myoblasts in culture (Wells and Strickland, Development, (1994), 120: 3639-3647)), a process that is inhibited by TGFb. TGFb exists as an inactive pro-polypeptide which is activated by limited proteolysis. The mechanism of aprotinin action has been proposed to involve inhibition of proteases which process pro-TGFb to the mature active form. TGFb has been shown to be up-regulated in various fibrotic lesions and has long thought to be a potential target for anti-fibrotic therapies. In a rat model of pulmonary fibrosis for example, TGF-b concentrations paralleled the extent of bleomycin-induced inflammation. Furthermore, plasmin levels in the alveolar macrophage coincided with mature TGF-b levels, and the addition of the plasmin inhibitor

a-2-antiplasmin abrogated the post translational activation of pro-TGFb by the macrophage (Khal et al., (1996), Am. J. Respir. Cell Mol. Biol. 15: 252-259.) The data suggest that plasmin contributes to the formation of active TGFb by alveolar macrophage, and that this process plays a pathologic role in the bleomycin-induced lung inflammation.

In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various fibrotic disorders, including pulmonary, hepatic, renal and dermal (scleroderma) fibrosis.

Aerosilized aprotinin was shown to protect >50% of mice infected with lethal doses of either influenza virus or paramyxovirus (Ovcharenko and Zhirnov, Antiviral Research, (1994), 23: 107-118). A suppression of the development of fatal hemorrhagic bronchopneumonia and a normalization of body weight gain were also noted with aerosilized aprotinin treatment. In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various respiratory related influenza-like diseases.

The human placental bikunin, isolated domains, and other variants of the invention are contemplated for use in the medical/therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitory profiles, in particular those which necessitate usage of large doses. These would include diseases for which use of the human protein is indicated by virtue of its ability to inhibit human serine proteases such as trypsin, plasmin, kallikrein, elastase, cathepsin G and proteinase-3, which include and are not limited to: acute pancreatitis (pancreatic elastase and trypsin), inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, various forms of shock, including shock lung, endotoxin shock and post operative complications; disturbances of blood coagulation such as hyperfibrinolytic hemorrhage; acute and chronic inflammatory reactions, in particular for the therapy and prophylaxis of organ lesions, such as for example pancreatitis and radiation induced enteritis, complex-mediated inflammatory reactions such as immunovasculitis, glomerulonephritis and types of arthritis; collagenoses in particular rheumatoid arthritis; types of arthritis caused by metabolism-related deposits (for example gout); degeneration of the elastic constituents of the connective tissue parts of organs, such as in atherosclerosis (serum elastase) or pulmonary emphysema (neutrophil elastase); adult respiratory distress syndrome, inflammatory bowel disease, and psoriasis.

A major unexpected finding was that the synthetic peptides encoding

bikunin (7-64), and bikunin (102-159), could properly fold into the correct three-dimensional conformation having active protease inhibitor bioactivity (Examples 2 and 1, respectively). Upon folding, each of these fragments of Bikunin underwent a reduction in mass of 6 mass units, consistent with the formation in each case, of three intrachain disulfide bonds between six cysteine residues of each fragment. Another surprising finding is that the synthetic peptides encoding bikunin (7-64), bikunin (102-159), and bikunin (1-170) are highly inhibitory of plasmin and both tissue and plasma kallikrein (Example 4 , 3, and 10 respectively). Inhibition of plasmin and kallikrein by Trasylol® is thought to be involved in the mechanism by which Trasylol® reduces blood loss during open heart surgery. Our unexpected findings of the specificity of the Kunitz domains of the present invention make them suitable therapeutic agents for blood sparing during surgery or trauma where there is significant blood loss, or for any other condition where inhibition of plasmin and/or kallikrein would be beneficial.

Furthermore, we showed in this disclosure (Example 10) that placental bikunin (1-170) is a potent inhibitor of factor XIa and a moderate inhibitor of factor Xa. Factor XIa plays an essential role in the intrinsic pathway of coagulation, serving to interconvert inactive factor IX into active factor IXa. Thus, Placental Bikunin inhibits two key enzymes of the intrinsic pathway, kallikrein and factor XIa. Consistent with these observations, we also showed that placental bikunin (1-170) is a potent inhibitor of the activated partial thromboplastin time, which is a measure of the speed of coagulation driven by the intrinsic pathway. On the other hand, we showed that Placental bikunin (1-170) is an extremely weak inhibitor of the tissue factor VIIa complex, suggesting that it is not important in the regulation of the extrinsic coagulation cascade. Based on these unexpected findings, placental bikunin is contemplated as a medicament for diseases in which activation of the intrinsic pathway of coagulation contributes significantly to the disease mechanism. Examples of such diseases would include post-traumatic shock and disseminated intravascular coagulation.

A significant advantage of the Kunitz domains of the present invention is that they are human proteins, and also less positively charged than Trasylol® (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly reduced risk of undesired immunological reactions as compared to

administration of similar doses of Trasylol®. Furthermore, it was found that bikunin (102-159), bikunin (7-64), and bikunin (1-170) are significantly more potent inhibitors of plasma kallikrein than Trasylol® *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* at lowering blood loss in patients.

The amount of serine protease inhibitor administered should be sufficient to provide a supra normal plasma level. For the prophylactic reduction of bleeding during and following coronary aortic by-pass surgery (CABG), the proteins of the instant invention may be used in place of Trasylol® while taking into account the differences in potency. The use of Trasylol® is outlined in the Physicians Desk Reference, (1995), listing for Trasylol® supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given slowly over about 20 to 30 minutes, after induction of anesthesia but prior to sternotomy. In general, a total dose of between about $2x10^6$ KIU (kallikrein inhibitory units) and $8 X10^6$ KIU will be used, depending on such factors as patient weight and the length of the surgery. Preferred loading doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU). When the loading dose is complete, it is followed by the constant infusion dose, which is continued until surgery is complete and the patient leaves the operating room. Preferred constant infusion doses are in the range of about 250,000 to 500,000 KIU per hour. The pump prime dose is added to the priming fluid of the cardiopulmonary bypass circuit, by replacement of an aliquot of the priming fluid prior to the institution of the cardiopulmonary bypass. Preferred pump prime doses are those that contain a total of about one to two million KIU.

The proteins of the instant invention are employed in pharmaceutical compositions formulated in the manner known to the art. Such compositions contain active ingredient(s) plus one or more pharmaceutically acceptable carriers, diluents, fillers, binders, and other excipients, depending on the administration mode and dosage form contemplated. Examples of therapeutically inert inorganic or organic carriers known to those skilled in the art include, but are not limited to, lactose, corn starch or derivatives thereof, talc, vegetable oils, waxes, fats, polyols such as polyethylene glycol, water, saccharose, alcohols, glycerin and the like. Various preservatives, emulsifiers, dispersants, flavorants, wetting agents, antioxidants, sweeteners, colorants, stabilizers, salts, buffers and the like can also be added, as required to assist in the stabilization of the formulation or to assist in increasing bioavailability of

the active ingredient(s) or to yield a formulation of acceptable flavor or odor in the case of oral dosing. The inhibitor employed in such compositions may be in the form of the original compound itself, or optionally, in the form of a pharmaceutically acceptable salt. The proteins of the instant invention can be adminstered alone, or in various combinations, and in combination with other therapeutic compositions. The compositions so formulated are selected as needed for administration of the inhibitor by any suitable mode known to those skilled in the art.

Parenteral administration modes include intravenous (*i.v.*), subcutaneous (*s.c.*), intraperitoneal (*i.p.*), and intramuscular (*i.m.*) routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed. Alternatively, the drug can be administered at a desired rate continuously by *i.v.* catheter. Suitable vehicles include sterile, non-pyrogenic aqueous diluents, such as sterile water for injection, sterile-buffered solutions or sterile saline. The resulting composition is administered to the patient prior to and/or during surgery by intravenous injection or infusion.

Improved half-life and targeting of the drug to phagosomes such as neutrophils and macrophage involved in inflammation may be aided by entrapment of the drug in liposomes. It should be possible to improve the selectivity of liposomal targeting by incorporating into the outside of the liposomes ligands that bind to macromolecules specific to target organs/tissues such as the GI tract and lungs. Alternatively, *i.m.* or *s.c.* deposit injection with or without encapsulation of the drug into degradable microspheres (e.g., comprising poly-DL-lactide-co-glycolide) or protective formulations containing collagen can be used to obtain prolonged sustained drug release. For improved convenience of the dosage form it is possible to use an *i.p.* implanted reservoir and septum such as the percuseal system. Improved convenience and patient compliance may also be achieved by use of either injector pens (e.g., the Novo Pin or Q-pen) or needle-free jet injectors (e.g., from Bioject, Mediject or Becton Dickinson). Precisely controlled release can also be achieved using implantable pumps with delivery to the desired site via a cannula. Examples include the subcutaneously implanted osmotic pumps available from ALZA such as the ALZET osmotic pump.

Nasal delivery may be achieved by incorporating the drug into bioadhesive particulate carriers (<200 mm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption

enhancers such as phospholipids or acylcarnitines. Commercially available systems include those developed by Dan Biosys and Scios Nova.

Pulmonary delivery represents a nonparenteral mode of administration of the drug to the circulation. The lower airway epithelia are highly permeable to a wide range of proteins of molecular sizes up to about 20 kDa. Micron-sized dry powders containing the medicament in a suitable carrier such as mannitol, sucrose or lactose may be delivered to the distal alveolar surface using dry powder inhalers such as those of Inhale™, Dura™, Fisons (Spinhaler™), and Glaxo (Rotahaler™), or Astra (Turbohaler™) propellant based metered dose inhalers. Solution formulations with or without liposomes may be delivered using ultrasonic nebulizers.

Oral delivery may be achieved by incorporating the drug into tablets, coated tablets, dragées, hard and soft gelatin capsules, solutions, emulsions, suspensions or enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples of the latter include the OROS-CT/Osmet™ system of ALZA, and the PULSINCAP™ system of Scherer Drug Delivery Systems. Other systems use azo-crosslinked polymers that are degraded by colon-specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH in the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers. Rectal delivery may be achieved by incorporating the drug into suppositories.

In its preferred medicinal application, for reduction of perioperative blood loss, the preferred mode of administration of the placental bikunin variants of the present invention is parenterally, preferably by *i.v.* route through a central line.

The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, based on a determination of the amount of target protease such as plasmin or kallikrein which must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent.

Additionally, placental bikunin, isolated domains or other variants may be used to isolate natural substances such as its cognate proteases from human

material using affinity based separation methods, as well as to elicit antibodies to the protease that can be further used to explore the tissue distribution and useful functions of Placental bikunin.

Searching Human Sequence Data

The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI (National Center for Biological Information, Maryland). Using the TBlastN algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215: 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, Trasylol®. This search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library. The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the longest translated open reading frame from clone R74593 (SEQ ID NO: 15) contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been sequenced incorrectly.

Discovery of Human Bikunin

To specifically isolate and determine the actual human sequence, cDNA primers were designed to be capable of hybridizing to sequences located 5' and 3' to the segment of cDNA encoding our proposed Kunitz like sequences found within R35464 and R74593. The primers used to amplify a fragment encoding the Kunitz like sequence of R74593 were:

CGAAGCTTCATCTCCGAAGCTCCAGACG (the 3'primer with a HindIII site; SEQ ID NO:33) and AGGATCTAGACAATAATTACCTGACCAAGGA (the 5'primer with an XbaI site; SEQ ID NO:34).

These primers were used to amplify by PCR (30 cycles) a 500 base pair product from a human placental cDNA library from Clontech (MATCHMAKER, Cat #HL4003AB, Clontech Laboratories, Palo Alto, CA), which was subcloned into Bluescript-SK+ and sequenced with the T3 primer with a Sequenase™ kit version 2.0. Surprisingly, the sequence of the fragment obtained using our primers was different from the sequence listed in the dbEST data base for clone R74593. In particular, our new sequence contained an additional guanosine base inserted 3' to the putative stop codon, but 5' to the segment encoding the Kunitz-like sequence (Figure 3). The insertion of an additional G shifted the stop codon out of the reading frame for the Kunitz-like domain (G at base pair 114 of the corrected sequence for R74593; Figure 3).

Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it contained all six of the characteristic cysteines. Overlay of each of the nucleotide sequences with that of R74593 (corrected by the insertion of G at b,p, 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3; full translation SEQ ID NO: 10) that contained two complete Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

Further efforts attempted to obtain additional 5' sequence by querying dbEST with the sequence of R35464. Possible matches from such searches, that possessed additional 5' sequence were then in turn used to re-query the dbEST. In such an iterative fashion, a series of overlapping 5' sequences were identified which included clones H16866, T66058, R34808, R87894, N40851 and N39876 (Figure 4). Alignment of some of these sequences suggested the presence of a 5' ATG which might serve as a start site for synthesis of the consensus translated protein sequence. From this selected information, it was now possible to selectively screen for, and determine the nucleic acid and polypeptide sequences of a human protein with homologous function to aprotinin.

Re-interrogation of the dbEST revealed a number of new EST entries

shown schematically in Figure 4B. Overlap with these additional ESTs allowed us to construct a much longer consensus oligonucleotide sequence (Figure 4C) that extended both 5' and 3' beyond the original oligonucleotide sequence depicted in Figure 3. In fact, the new sequence of total length 1.6 kilobases extended all the way to the 3' poly-A tail. The increased number of overlapping ESTs at each base-pair position along the sequence improved the level of confidence in certain regions such as the sequence overlapping with the 3' end of EST R74593 (Figure 3). Several overlapping ESTs in this region corroborated two critical base deletions relative to R74593 (located as bold underlined in Figure 4C, map positions 994 and 1005). Translation of the new consensus sequence (Figure 4D) in the bikunin encoding frame yielded a form of placental bikunin that was larger (248 amino acids) than the mature sequence (179 amino acids) encoded from the original consensus (SEQ ID NO: 1), and was terminated by an in-frame stop codon within the oligonucleotide consensus. The size increase was due to a frame shift in the 3' coding region resulting from removal of the two base insertions unique to EST R74593. The frame shift moved the stop codon of the original consensus (Figure 3) out of frame enabling read through into a new frame encoding the additional amino acid sequence. The new translation product (Figure 4D) was identical to the original protein consensus sequence (SEQ ID NO: 1) between residues +1 to +175 (encoding the Kunitz domains), but contained a new C-terminal extension exhibiting a putative 24 residue long transmembrane domain (underlined in Figure 4D) followed by a short 31 residue cytoplasmic domain. The precise sequence around the initiator methionine and signal peptide was somewhat tentative due to considerable heterogeneity amongst the overlapping ESTs in this region.

Analysis of the protein sequence by Geneworks™, highlighted asparagine residues at positions 30 and 67 as consensus sites for putative N-linked glycosylation. Asparagine 30 was not observed during N-terminal sequencing of the full length protein isolated from human placenta, consistent with it being glycosylated.

Cloning of Human Bikunin

The existence of a human mRNA corresponding to the putative human bikunin nucleotide sequence inferred from the analysis of Figure 3, was confirmed as follows. The nucleic acid primer hybridizing 5' to the Kunitz-encoding cDNA sequence of R35464 (b.p. 3-27 of consensus nucleotide

sequence in Figure 3):

GGTCTAGAGGCCGGGTCGTTTCTCGCCTGGCTGGGA

(a 5' primer derived from R35464 sequence with an XbaI site; SEQ ID NO: 35), and the nucleic acid primer hybridizing 3' to the Kunitz encoding sequence of R74593 (b.p. 680-700 of consensus nucleotide sequence in Figure 3), was used to PCR amplify, from a Clontech human placental library, a fragment of the size (ca. 670 b.p) expected from a cDNA consensus nucleotide sequence encoding the placental bikunin sequence of Figure 3 (Shown schematically in Figure 4A).

Using a 5' primer hybridizing to a sequence in R87894 that is 126 b.p 5' to the putative ATG start site discussed above, (shown schematically in Figure 4A at b.p. 110) plus the same 3' primer to R74593 as used above, it was possible to amplify a fragment from a Clontech human placental library of the expected size (approximately 872 b.p) predicted by EST overlay (Shown schematically in Figure 4).

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5' end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3' end. This 3' nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159).

To obtain a cDNA encoding the entire extracellular region of the protein, the following 5' PCR primer:

CACCTGATCGCGAGACCCC (SEQ ID NO: 36)

designed to hybridize to a sequence within EST R34808 was used with the same 3' primer to EST 74593 to amplify (30 cycles) an approximately 780 base-pair cDNA product from the human placental cDNA library. This product was gel purified, and cloned into the TA vector (Invitrogen) for DNA sequencing by the dideoxy method (Sanger F., et al., (1977) Proc. Natl. Acad. Sci (USA), 74: 5463-5467) with the following primers:

Vector Specific: GATTTAGGTGACACTATAG (SP6) (SEQ ID NO: 37)
TAATACGACTCACTATAGGG (T7) (SEQ ID NO: 38)

Gene Specific: TTACCTGACCAAGGAGGAGTGC (SEQ ID NO: 39)
AATCCGCTGCATTCCTGCTGGTG (SEQ ID NO: 40)
CAGTCACTGGGCCTTGCCGT (SEQ ID NO: 41)

The resulting cDNA sequence is depicted in Figure 4E together with its

translation product. At the nucleotide level, the sequence exhibited only minor differences from the consensus EST sequence (Figure 4D). Translation of the sequence yielded a coding sequence containing an in-frame initiator ATG site, signal peptide and mature placental bikunin sequence and transmembrane domain. The translated sequence of the PCR product was missing the last 12 amino acid residues from the cytoplasmic domain as a consequence of the choice of selection of the 3' primer for PCR amplification. This choice of 3' PCR primer (designed based on the sequence of R74593) was also responsible for the introduction of an artifactual S to F mutation at amino acid position 211 of the translated PCR-derived sequence. The signal peptide deduced from translation of the PCR fragment was somewhat different to that of the EST consensus.

To obtain a full length placental bikunin cDNA, the PCR derived product (Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with ^{32}P-CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, Unizap™ λ library) using colony hybridization techniques. Approximately 2 X 10^6 phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7). Both the natural protein and the form of the protein expressed in Sf9 cells are probably glycosylated at the asparagine

residue at position 30 based on the recoveries of PTH-amino acids during N-terminal sequencing (Examples 7 and 9).

Based on the above observations, it seems that full length placental bikunin has the capacity to exist as a transmembrane protein on the surface of cells as well as a soluble protein. Other transmembrane proteins that contain Kunitz domains are known to undergo proteolytic processing to yield mixtures of soluble and membrane associated forms. These include two forms of the Amyloid Precursor Protein termed APP751 (Esch F., et al., (1990) Science, 248: 1122-1124) and APP 770 (Wang R., et al., (1991), J. Biol Chem, 266: 16960-16964).

Contact activation is a process which is activated by exposure of damaged vascular surfaces to components of the coagulation cascade. Angiogenesis is a process that involves local activation of plasmin at endothelial surfaces. The specificity of placental bikunin and its putative capacity to anchor to cell surfaces, suggest that the physiologic functions of transmembranous placental bikunin may include regulation of contact activation and angiogenesis.

The amino acid sequences for placental bikunin (7-64), bikunin (102-159), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFastA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85: 2444-2448), these same protein sequences were searched versus the six-frame translations of the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL (modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

Isolation of Human Bikunin

As mentioned above, synthetic peptides corresponding to bikunin (7-64) and bikunin (102-159) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate

native placental bikunin from human tissue.

Using a purification scheme which employed kallikrein-sepharose affinity chromatography as a first step, highly purified native potent kallikrein inhibitor was isolated. The isolated native human bikunin had an identical N-terminus (sequenced for 50 amino acid residues) as the sequence predicted by the translation of the consensus nucleic acid sequence (Figure 3) amino acid residues +1 to +50 (Example 7). This confirmed for the first time the existence of a novel native kallikrein inhibitor isolated from human placenta.

Known Kunitz-like domains are listed below. Residues believed to be making contact with target proteases are highlighted as of special interest (bold/underlined). These particular residues are named positions Xaa$^{1-16}$ for specific reference as shown by label Xaa below:

```
     Xaa
      1          2   3 456789                   1  1   111      1         1
                                                0  1   234      5         6
 1)  IHDFCLVSKVV GRCRASMPRW WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATV
 2)  YEEYCTANAVT GPCRASFPRW YFDVERNSCN NFIYGGCRGN KNSYRSEEAC MLRCFRQ
 3)  -HSFCAFKADD GPCKAIMKRF FFNIFTRQCE EFIYGGCEGN QNRFESLEEC KKMCTRD
 4)  -PDFCFLEEDP GICRGYITRY FYNNQTKQCE RFKYGGCLGN MNNFETLEEC KNICEDG
 5)  -PSWCLTPADR GLCRANENRF YYNSVIGKCR PFKYSGCGGN ENNFTSKQEC LRACKKG
 6)  -AEICLLPLDY GPCRALLLRY YYRYRTQSCR QFLYGGCEGN ANNFYTWEAC DDACWRI
 7)  -PSFCYSPKDE GLCSANVTRY YFNPRYRTCD AFTYTGCGGN DNNFVSREDC KRACAKA
 8)  -KAVCSQEAMT GPCRAVMPRT TFDLSKGKCV RFITGGCGGN RNNFESEDYC MAVCKAM
 9)  RPDFCLEPPYT GPCKARIIRY FYNAKAGLCQ TFVYGGCRAK RNNFKSAEDC MRTCGGA
10)  ----CQLGYSA GPCMGMTSRY FYNGTSMACE TFQYGGCMGN GNNFVTEKEC LQTC
11)   VAACNLPIVR GPCRAFIQLW AFDAVKGKCV LFPYGGCQGN GNKFYSEKEC REYCGVP
12)  -EVCCSEQAET GPCRAMISRW YFDVTEGKCA PFFYGGCGGN RNNFDTEEYC MAVCGSA
13)  ----CKLPKDE GTCRDFILKW YYDPNTKSCA RFWYGGCGGN ENKFGSQKEC EKVC
14)  -PNVCAFPMEK GPCQTYMTRW FFNFETGECE LFAYGGCGGN SNNFLRKEKC EKFCKFT
```

Where sequence number 1) is Bikunin (7-64) (SEQ ID NO: 4); sequence 2) is Bikunin (102-159) (SEQ ID NO: 6); sequence 3) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 18); sequence 4) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 19); sequence 5) is Tissue factor pathway inhibitor precursor (SEQ ID NO: 20); sequence 6) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 21); sequence 7) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 22); sequence 8) is Amyloid precursor protein homologue (SEQ ID NO: 23); sequence 9) is Aprotinin (SEQ ID NO: 24);

sequence 10) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 25); sequence 11) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 26); sequence 12) is Amyloid precursor protein (SEQ ID NO: 27); sequence 13) is Collagen α-3(VI) precursor (SEQ ID NO: 28); and squence 14) is HKI-B9 (SEQ ID NO: 29).

It can be seen that Placental Bikunin (7-64) and (102-159) each have the same number (six) and spacing of cysteine residues as is found in members of the Kunitz class of serine protease inhibitors. The precise bonding of cysteine residues to form the three intrachain disulfide bonds is known and invarient for all previously known Kunitz family members (Laskowski, M et al., 1980, Ann. Rev. Biochem. 49:593-626). Based on this known bonding pattern and the fact that the folding of Placental Bikunin (7-64) and (102-159) into active protease inhibitors is accompanied by a mass reduction consistent with the formation of three intrachain disulfide bonds (Examples 2 and 1), it is highly probable that the disulfide bonding within the Kunitz domains of Placental Bikunin occur between cysteine residues: C11 and C61; C20 and C44; C36 and C57; C106 and C156; C115 and C139; C131 and C152. Furthermore, this pattern of disulfide bonding is highly probable in larger forms of Placental Bikunin containing both Kunitz domains since such forms of the protein are also active serine protease inhibitors and because N-terminal sequencing (Example 7) of native Placental Bikunin for 50 cycles yielded a sequence that was silent at positions where the cysteine residues were expected.

The placental bikunin, isolated domains or other variants of the present invention may be produced by standard solid phase peptide synthesis using either t-Boc chemistry as described by Merrifield R.B. and Barany G., in: The peptides, Analysis, Synthesis, Biology, 2, Gross E. et al., Eds. Academic Press (1980) Chapter 1; or using F-moc chemistry as described by Carpino L.A., and Han G.Y., (1970) J. Amer Chem Soc., 92, 5748-5749, and illustrated in Example 2. Alternatively, expression of a DNA encoding the placental bikunin variant may be used to produce recombinant placental bikunin variants.

The invention also relates to DNA constructs that encode the Placental bikunin protein variants of the present invention. These constructs may be prepared by synthetic methods such as those described in Beaucage S.L. and Caruthers M.H., (1981) Tetrahedron Lett, 22, pp1859-1862; Matteucci M.D and Caruthers M.H., (1981), J. Am. Chem. Soc. 103, p 3185; or from genomic or cDNA which may have been obtained by screening genomic or cDNA libraries with cDNA probes designed to hybridize with placental bikunin encoding DNA sequence. Genomic or cDNA sequence can be modified at one or more

sites to obtain cDNA encoding any of the amino acid substitutions or deletions described in this disclosure.

The instant invention also relates to expression vectors containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. The cDNA should be connected to a suitable promoter sequence which shows transcriptional activity in the host cell of choice, possess a suitable terminator and a poly-adenylation signal. The cDNA encoding the placental bikunin variant can be fused to a 5' signal peptide that will result in the protein encoded by the cDNA to undergo secretion. The signal peptide can be one that is recognized by the host organism. In the case of a mammalian host cell, the signal peptide can also be the natural signal peptide present in full length placental bikunin. The procedures used to prepare such vectors for expression of placental bikunin variants are well known in the art and are for example described in Sambrook et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, New York, (1989).

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as E. coli, and yeasts such as Saccharomyces cervisiae. Methods for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., Current Protocols in Molecular Biology, John Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) and (102-159), yeast and E. coli expression systems are preferable, with yeast systems being most preferred. Typically, yeast expression would be carried out as described in US patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159). E.coli expression could be carried out using the methods described in US patent 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin (7-159).

DNA encoding variants of placental bikunin that possess amino acid substitution of the natural amino sequence can be prepared for expression of recombinant protein using the methods of Kunkel T.A., (1985) Proc. Natl. Acad. Sci USA 82: 488-492. Briefly, the DNA to be mutagenized is cloned into a single stranded bacteriophage vector such as M13. An oligonucleotide spanning the region to be changed and encoding the substitution is hybridized to the single stranded DNA and made double stranded by standard molecular biology techniques. This DNA is then transformed into an appropriate bacterial host and verified by dideoxynucleotide sequencing. The correct DNA is then cloned into the expression plasmid. Alternatively, the target DNA may be mutagenized by standard PCR techniques, sequenced, and inserted into the appropriate expression plasmid.

The following particular examples are offered by way of illustration, and not limitation, of certain aspects and preferred embodiments of the instant invention.

Example 1

Preparation of synthetic placental bikunin (102-159)

Materials and methods/Reagents used. The fluorogenic substrate Tos-Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC, bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

Recombinant aprotinin (Trasylol®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich (Milwaukee, WI).

Quantification of functional placental bikunin (7-64) and (102-159)

The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37°C with bikunin (7-64) or (102-159), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM $CaCl_2$ and 0.01% triton X-100). GPK-AMC was added (20 μM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition

for each was calculated according to equation 1; where R_o is the rate of fluorescence increase in the presence of inhibitor and R_1 is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.

$$\% \text{ inhibition} = 100 \times [1 - R_o/R_1] \quad (1)$$

Synthesis. Placental bikunin (102-159) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of amino acid for each coupling. Cleavage and deprotection was performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H_2O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy (MH+ =6836.1; calcd = 6835.5) for the predicted sequence:

YEEYCTANAV TGPCRASFPR WYFDVERNSC NNFIYGGCRG NKNSYRSEEA CMLRCFRQ (SEQ ID NO: 6)

Purification. Refolding of placental bikunin (102-159) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113: 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0. to obtain a final concentration of 0.5 mg/ml peptide in 20% DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The

column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active fractions were pooled (see below) and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitrile in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized, redissolved in 0.1% TFA, and stored at -20°C until needed.

Results. Synthetic placental bikunin (102-159) was refolded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

Table 1

Purification table for the isolation of synthetic placental bikunin (102-159)

TABLE 1

Purification Step	Vol (ml)	mg/ml	mg	Units[c] (U)	SpA (U/mg)	Yield
8.0 M Urea	4.0	3.75[a]	15.0	0	0	-
20% DMSO	32.0	0.47[a]	15.0	16,162	1,078	100
Kallikrein affinity	9.8	0.009[b]	0.09	15,700	170,000	97
C18	3.0	0.013[ab]	0.04	11,964	300,000	74

[a]Protein determined by AAA.
[b]Protein determined by OD280 nm using the extinction coefficient determined for the purified protein (1.7×10^4 $Lmol^{-1}$ cm^{-1}).
[c]One Unit is defined as the amount of material required to inhibit 50% of trypsin activity in a standard assay.

Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast Ampholine® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) was determined to be 8.3, in agreement with the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 152: 33-50).

Example 2

Preparation of synthetic placental bikunin (7-64)

Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

Results. The final purified reduced peptide exhibited an MH+ = 6563, consistent with the sequence:

IHDFCLVSKV VGRCRASMPR WWYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was active as an inhibitor of trypsin (Table 2 below).

Table 2A

Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2A

Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	8.0	2.5	20.0	0	0	-
20% DMSO	64.0	0.31	20.0	68,699	3,435	100
Kall affinity pH 4.0	11.7	0.10	1.16	43,333	36,110	62
Kall affinity pH 1.7	9.0	0.64	5.8	4972	857	7.2
C18-1	4.6	0.14	0.06	21,905	350,143	31.9
C18-2	1.0	0.08	0.02	7,937	466,882	11.5

The purified refolded protein exhibited an MH+ = 6558, i.e. 5±1 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of at least one appropriate disulfide bond.

The pI of placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Placental bikunin (7-64) exhibited a pI that was much higher than the predicted value (pI = 7.9). Refolded placental bikunin (7-64) migrated to the cathodic edge of the gel (pH 9.5) and an accurate pI could not be determined under these conditions.

Continued Preparation of synthetic placental bikunin (7-64)

Because the synthetic placental bikunin (7-64) may not have undergone complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide (0.27 mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25 C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

Results. The final purified reduced peptide exhibited an MH+ = 6567.5 , consistent with the sequence:

IHDFCLVSKV VGRCRASMPRW WYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was as active as an inhibitor of trypsin (Table 2B below).

Table 2B
Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2B

Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	4.9	2.1	10.5	0	0	-
20% DMSO	39.0	0.27	10.5	236,000	22,500	100
Kallikrein Affinity (pH 2)	14.5	0.3	0.43	120,000	279,070	50.9
C18 Reverse-Phase	0.2	1.2	0.24	70,676	294,483	30.0

The purified refolded protein exhibited an MH+ = 6561.2, i.e. 6.3 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of the expected three disulfide bonds.

The pI of refolded placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Refolded placental bikunin (7-64) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

Example 3

In vitro specificity of functional placental bikunin fragment (102-159)

Proteases. Bovine trypsin, human plasmin, and bovine pancreatic kallikrein quantitation was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase,T., and Shaw, E., (1970) Methods Enzmol., 19: 20-27). Human kallikrein was quantitated by active site titration using bovine aprotinin as a standard and PFR-AMC as a substrate assuming a 1:1 complex formation. The K_m for GPK-AMC with trypsin and plasmin under the conditions used for each enzyme was 29 µM and 726 µM, respectively; the K_m for PFR-AMC with human plasma kallikrein and bovine pancreatic kallikrein was 457 µM and 81.5 µM, respectively; the K_m for AAPR-AMC with elastase was 1600 µM. Human tissue kallikrein (Bayer, Germany) quantification was carried out by active site titration using p nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) Methods Enzmol. 19: 20-27).

Inhibition Kinetics: The inhibition of trypsin by placental bikunin (102-159) or aprotinin was measured by the incubation of 50 pM trypsin with

placental bikunin (102-159) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15 µl of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25 µl of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at 37°C 15 µl of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100 µM. The apparent inhibition constant K_i^* was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): The kinetic data from each experiment were analyzed in terms of the equation for a tight binding inhibitor:

$$V_i/V_o = 1 - (E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4\,E_o I_o]^{1/2})/2E_o \qquad (2)$$

where V_i/V_o is the fractional enzyme activity (inhibited vs. uninhibited rate), and E_o and I_o are the total concentrations of enzyme and inhibitor, respectively. Ki values were obtained by correcting for the effect of substrate according to the equation:

$$K_i = K_i^*/(1 + [S_o]/K_m) \qquad (3)$$

(Boudier, C., and Bieth, J. G., (1989) Biochim Biophys Acta., 995: 36-41)

For the inhibition of human neutrophil elastase by placental bikunin (102-159) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (150 nM) or aprotinin (0-7.5 µM) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37%C, AAPM-AMC (500 µM or 1000 µM) was added and the fluorescence measured over a two-minute period. Ki values were determined from Dixon plots of the form 1/V versus [I]

performed at two different substrate concentrations (Dixon et al., 1979).

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) or placental bikunin fragment (102-159) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (0-40 nM) or placental bikunin (102-159) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10 uM final and the change in fluorescence monitored. The Km for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7 uM. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at 37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64), 0 to 140 nM placental bikunin (102-159) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl2, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

Results: A direct comparison of the inhibition profiles of placental bikunin (102-159) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K_i values are listed in Table 3 below.

Table 3
Ki values for the inhibition of various proteases by bikunin (102-159)

TABLE 3

Protease (concentration)	bikunin (102-159) Ki (nM)	Aprotinin Ki (nM)	Substrate (concentration)	Km (mM)
Trypsin (48.5 pM)	0.4	0.8	GPK-AMC (0.03 mM)	0.022
Chymotrypsin (5 nM)	0.24	0.86	AAPF-pNA (0.08 mM)	0.027
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	PFR-AMC (0.1 mM)	0.08
Human Plasma Kallikrein (2.5 nM)	0.3	19.0	PFR-AMC (0.3 mM)	0.46
Human Plasmin (50 pM)	1.8	1.3	GPK-AMC (0.5 mM)	0.73
Human Neutrophil Elastase (19 nM)	323.0	8500.0	AAPM-AMC (1.0 μM)	1.6
Factor XIIa	>300.0	12,000.0	PFR-AMC (0.2 μM)	0.35
Human Tissue Kallikrein (0.35 nM)	0.13	0.004	PFR-AMC (10 μM)	0.0057
factor Xa (0.87 nM)	274	N.I. at 3 μM	LGR-AMC (0.6 mM)	N.D.
urokinase	11000	4500	GGR-AMC (0.7 mM)	N.D.
factor XIa (0.1 nM)	15	288	E(OBz)AR-AMC (0.4 mM)	0.46

Placental bikunin (102-159) and aprotinin inhibit bovine trypsin and human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a Ki of 8.5 μM. Placental bikunin (102-159) inhibited elastase with a Ki of 323nM. The K_i value for the placental bikunin (102-159) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) is a more potent inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

Because placental bikunin (102-159) is greater than 50 times more potent than Trasylol® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159)) are needed than Trasylol® in order to maintain the effective patient doses of inhibitor in KIU. This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients. Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

Example 4

In vitro specificity of functional placental bikunin fragment (7-64)

In vitro specificity of functional human placental bikunin (7-64) was determined using the materials and methods as described in the Examples above.

Results: The table below shows the efficacy of placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4 A
Ki values for the inhibition of various proteases by bikunin(7-64)

TABLE 4A

Protease (concentration)	bikunin(7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (48.5 pM)	0.17	0.8	0.4
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	0.4
Human Plasma Kallikrein (2.5 nM)	2.4	19.0	0.3
Human Plasmin (50 pM)	3.1	1.3	1.8
Bovine chymotrypsin (5 nM)	0.6	0.9	0.2
Factor XIIa	>300	12000	>300
elastase	>100	8500	323

The results show that the amino acid sequence encoding placental bikunin (7-64) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

Table 4B below also shows the efficacy of refolded placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4B
Ki values for the inhibition of various proteases by refolded bikunin (7-64)

TABLE 4B

Protease (concentration)	bikunin (7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (50 pM)	0.2	0.8	0.3
Human Plasma Kallikrein (0.2 nM)	0.7	19.0	0.7
Human Plasmin (50 pM)	3.7	1.3	1.8
Factor XIIa	not done	12,000	4,500
Factor XIa (0.1 nM)	200	288	15
Human Tissue Kallikrein	2.3	0.004	0.13

Suprisingly, placental bikunin (7-64) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

Example 5

Expression of placental bikunin variant (102-159) in yeast

The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO: 6) was generated using synthetic oligonucleotides. The final DNA product consisted (5' to 3') of 15 nucleotides from the yeast α-mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast α-mating factor propeptide fused to the 58 amino acid sequence of placental bikunin (102-159). Processing of this fusion protein at a KEX-2 cleavage site at the junction between the α-mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

A 5' sense oligonucleotide of the following sequence and containing a HindIII site for cloning was synthesized:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC TTT GAC GTG GAG AGG (SEQ ID NO: 42)

A 3' antisense oligonucleotide of the following sequence and containing both a BamHI site for cloning and a stop codon was synthesized:

CGC GGA TCC CTA CTG GCG GAA GCA GCG GAG CAT GCA GGC CTC CTC AGA GCG GTA GCT GTT CTT ATT GCC CCG GCA GCC TCC ATA GAT GAA GTT ATT GCA GGA GTT CCT CTC CAC GTC AAA GTA CCA GCG
(SEQ ID NO: 43)

The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII and BamHI. The resulting digested double stranded fragment was cloned into pUC19 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC TTT GAC GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC TAT GGA GGC TGC CGG GGC AAT AAG AAC AGC TAC CGC TCT GAG GAG GCC TGC ATG CTC CGC TGC TTC CGC CAG TAG GGA TCC (SEQ ID.: 44)

which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transformed into yeast strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159).

Detection of expression of placental bikunin (102-159) in transformed yeast

Firstly, the supernatants (50 ul per assay) were evaluated for their capacity to inhibit the *in vitro* activity of trypsin using the assay methods as described in Example 1 (1 ml assay volume). An un-used media only sample as well as a yeast clone expressing an inactive variant of aprotinin served as

negative controls. A yeast clone expressing natural aprotinin served as a positive control and is shown for comparison.

The second method to quantify placental bikunin (102-159) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug of purified reduced synthetic placental bikunin (102-159), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 µl) were subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's directions (Kirkegaard and Perry, Gaithersburg, MD).

Purification of placental bikunin (102-159) from a transformed strain of SC101

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM $CaCl_2$ and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted fractions were pooled and applied to a C18 column (Vydac, 5um, 4.6 x 250 mm)

previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin (102-159) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

Results. Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159). The same 6 kDa band in the aprotinin control did not react with the same antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) while about 60 % of the material contained a portion of the yeast α-mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent Ki of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) in fermentation broth as well as the isolation of placental bikunin (102-159) from

one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159, as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (YEEY--) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P′ subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (defined as construct #1) but with the following modifications:

Construct #2 placental bikunin 103-159, yeast codon usage

A 5′ sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG CTAATGCTGT TACTGGTCCA TGTAGAGCTT CTTTTCCAAG ATGGTACTTT GATGTTGAAA GA (SEQ ID NO: 55)

and 3′ antisense oligonucleotide

ACTGGATCCT CATTGGCGAA AACATCTCAA CATACAGGCT TCTTCAGATC TGTAAGAATT TTTATTACCT CTACAACCAC CGTAAATAAA ATTATTACAA GAATTTCTTT CAACATCAAA GTACCATCT (SEQ ID NO: 56)

were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159

Construct #3 placental bikunin 101-159, yeast codon usage

A 5′ sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT GTACTGCTAA TGCTGTTACT GGTCCATGTA GAGCTTCTTT TCCAAGATGG TACTTTGATG TTGAAAGA (SEQ ID NO: 57)

and the same 3′ antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159.

Construct #4 placental bikunin 98-159, yeast codon usage

A 5′ sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGATATG TTTAATTACG AAGAATACTG TACTGCTAAT GCTGTTACTG GTCCATGTAG AGCTTCTTTT CCAAGATGGT ACTTTGATGT TGAAAGA (SEQ ID NO: 58)

and the same 3′ antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above).

Yeast strain SC101 (MATα, ura 3-52, suc 2) was transformed with the plasmids containing each of the above cDNAs, and proteins were expressed using the methods that were described above for the production of placental bikunin 102-159 with human codon usage. Approximately 250 ml of each yeast culture was harvested, and the supernatant from centrifugation (15 min x 3000 RPM) separately subjected to purification over 1 ml columns of kallikrein-sepharose as described above. The relative amount of trypsin inhibitory activity in the applysate, the amount of purified protein recovered and the N-terminal sequence of the purified protein were determined and are listed below in Table 7.

Table 7
Relative production levels of different proteins containing the C-terminal Kunitz domain of placental bikunin

TABLE 7

Construct		Relative conc. of inhibitor in applysate	N-terminal sequencing: amount (pmol)	sequence	Comments
#2	103-159	none detected	none	none	no expression
#3	101-159	25 % inhibition	none	none	low expression
#4	98-159	93 % inhibition	910	DMFNYE-	good expression correct product
#1	102-159	82 % inhibition	480	AKEEGV-	expression of active incorrectly processed protein

The results show that placental bikunin fragments of different lengths that contain the C-terminal Kunitz domain show wide variation in capacity to express functional secreted protein. Constructs expressing fragments 101-159 and 103-159 yielded little or low enzymic activity in the supernatants prior to purification, and N-terminal sequencing of 0.05 ml aliquots of each purified fraction yielded undetectable amounts of inhibitor. On the other hand expression either of placental bikunin 102-159 or 98-159 yielded significant amounts of protease activity prior to purification. N-terminal sequencing however showed that the purified protein recovered from expression of 102-159 was once again largely incorrectly processed, exhibiting an N-terminus consistent with processing of the majority of the pre-protein at a site within the yeast α-mating factor pro-sequence. The purified protein recovered from expression of placental bikunin 98-159 however was processed entirely at the correct site to yield the correct N-terminus. Furthermore, nearly twice as much protein was recovered as compared to the recovery of placental bikunin 102-159. Placental bikunin 98-159 thus represents a preferred fragment length for the production of the C-terminal Kunitz domain of placental bikunin by the α-mating factor pre-pro sequence/ KEX-2 processing system of *S. cerevisiae*,

Example 6

Alternative procedure for yeast expression

The 58 amino acid peptide derived from the R74593 translation product can also be PCR amplified from either the R87894-R74593 PCR product cloned into the TA vector™ (Invitrogen, San Diego, CA) after DNA sequencing or from human placental cDNA. The amplified DNA product will consist of 19 nucleotides from the yeast α-mating factor leader sequence mated to the R74593 sequence which codes for the YEEY--CFRQ (58 residues) so as to make the translation product in frame, constructing an α -mating factor/Kunitz domain fusion protein. The protein sequence also contains a kex 2 cleavage which will liberate the Kunitz domain at its native N-terminus.

The 5' sense oligonucleotide which contains a HindIII site for cloning will contain the following sequence:

GCCAAGCTTG GATAAAAGAT ATGAAGAAT ACTGCACCGC CAACGCA (SEQ ID NO: 30)

The 3' antisense oligonucleotide contains a BamHI site for cloning as well as a stop codon and is of the following sequence:

GGGGATCCTC ACTGCTGGCG GAAGCAGCGG AGCAT (SEQ ID NO: 31

The full 206 nucleotide cDNA sequence to be cloned into the yeast expression vector is of the following sequence:

CCAAGCTTGG ATAAAAGATA TGAAGAATAC TGCACCGCCA ACGCAGTCAC
TGGGCCTTGC CGTGCATCCT TCCCACGCTG GTACTTTGAC GTGGAGAGGA
ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC
TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGTGAGG
ATCCCC (SEQ ID NO: 32)

After PCR amplification, this DNA will be digested with HindIII, BamHI and cloned into the yeast expression vector pMT15 (see US patent 5,164,482, incorporated by reference in the entirety) also digested with HindIII and BamHI. The resulting plasmid vector is used to transform yeast strain SC 106 using the methods described in US patent 5,164,482. The URA 3- yeast transformants are isolated and cultivated under inducing conditions. The yield of recombinant Placental bikunin variants is determined according to the

amount of trypsin inhibitory activity that accumulated in the culture supernatants over time using the *in vitro* assay method described above. Fermentation broths are centrifuged at 9000 rpm for 30 minutes. The supernatant is then filtered through a 0.4 then a 0.2 μm filter, diluted to a conductivity of 7.5 ms, and adjusted to pH 3 with citric acid. The sample is then batch absorbed onto 200 ml of S-sepharose fast flow (Pharmacia) in 50 mM sodium citrate pH 3 and stirred for 60 min. The gel is subsequently washed sequentially with 2 L of each of: 50 mM sodium citrate pH 3.0; 50 mM Tris-HCL pH 9.0; 20 mM HEPES pH 6.0. The washed gel is transferred into a suitable column and eluted with a linear gradient of 0 to 1 M sodium chloride in 20 mM HEPES pH 6.0. Eluted fractions containing *in vitro* trypsin inhibitory activity are then pooled and further purified either by a) chromatography over a column of immobilized anhydrotrypsin (essentially as described in Example 2); b) by chromatography over a column of immobilized bovine kallikrein; or c) a combination of conventional chromatographic steps including gel filtration and/or anion-exchange chromatography.

Example 7
Isolation and characterization of native human placental bikunin from placenta

Bikunin protein was purified to apparent homogeniety from whole frozen placenta (Analytical Biological Services, Inc, Wilmington, DE). The placenta (740 gm) was thawed to room temperature and cut into 0.5 to 1.0 cm pieces, placed on ice and washed with 600 ml PBS buffer. The wash was decanted and 240 ml of placenta pieces placed into a Waring blender. After adding 300 ml of buffer consisting of 0.1 M Tris (pH 8.0), and 0.1 M NaCl , the mixture was blended on high speed for 2 min, decanted into 750.0 ml centrifuge tubes, and placed on ice. This procedure was repeated until all material was processed. The combined slurry was centrifuged at 4500 x g for 60 minutes at 4°C. The supernatant was filtered through cheese cloth and the placental bikunin purified using a kallikrein affinity column made by covalently attaching 70 mg of bovine pancreatic kallikrein (Bayer AG) to 5.0 mls of CNBr activated Sepharose (Pharmacia) according to manufacturers instruction. The material was loaded onto the affinity column at a flow rate of 2.0 ml/min and washed with 0.1 M Tris (pH 8.0), 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was further washed with 0.1 M Tris (pH 8.0), 0.5 M NaCl and then eluted with 3 volumes of 0.2 M acetic acid,

pH 4.0. Fractions containing kallikrein and trypsin inhibitory (see below) activity were pooled, frozen, and lyophilized. Placental bikunin was further purified by gel-filtration chromatography using a Superdex 75 10/30 (Pharmacia) column attached to a Beckman System Gold HPLC system. Briefly, the column was equilibrated in 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 at a flow rate of 0.5 ml/min. The lyophilized sample was reconstituted in 1.0 ml of 0.1 M Tris, pH 8.0 and injected onto the gel-filtration column in 200 µl aliquots. Fractions were collected (0.5 ml) and assayed for trypsin and kallikrein inhibitory activity. Active fractions were pooled, and the pH of the solution adjusted to 2.5 by addition of TFA. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 20% acetonitrile in 0.1 %TFA. Separation was achieved using a linear gradient of 20 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min over 50 minutes after an initial 20 minute wash at 20% acetonitrile in 0.1% TFA. Fractions (1ml) were collected and assayed for trypsin and kallikrein inhibitory activity. Fractions containing inhibitory activity were concentrated using a speed-vac concentrator (Savant) and subjected to N-terminal sequence analysis.

Functional assays for Placental Bikunin:

Identification of functional placental bikunin was achieved by measuring its ability to inhibit bovine trypsin and human plasma kallikrein. Trypsin inhibitory activity was performed in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2.0 mM CaCl2, 0.1% Triton x-100) at room temperature in a 96-well microtiter plate (Perkin Elmer) using Gly-Pro-Lys-Aminomethylcoumarin as a substrate. The amount of coumarin produced by trypsin was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter equipped with a plate reader. Trypsin (23 µg in 100 µl buffer) was mixed with 20 µl of the sample to be tested and incubated for 10 minutes at 25°C. The reaction was started by the addition of 50 µl of the substrate GPK-AMC (33 µM final) in assay buffer. The fluorescence intensity was measured and the % inhibition for each fraction was determined by:

$$\% \text{ inhibition} = 100 \times [1 - Fo/F1]$$

where Fo is the fluorescence of the unknown and F1 is the fluorescence of the trypsin only control. Kallikrein inhibitory activity of the fractions was similarly measured using 7.0 nM kallikrein in assay buffer (50 mM Tris, pH 8.0, 50 mM

NaCl, 0.1% triton x-100) and 66.0 μM Pro-Phe-Arg-AMC as a substrate.

Determination of the *in vitro* specificity of placental bikunin

The *In vitro* specificity of native human placental bikunin was determined using the materials and methods as described in the preceding examples above. Placental bikunin was quantified by active site titration against a known concentration of trypsin using GPK-AMC as a substrate to monitor the fraction of unbound trypsin.

Protein Sequencing

The 1 ml fraction (C18-29 Delaria) was reduced to 300 ml in volume, on a Speed Vac, to reduce the amount of organic solvent. The sample was then loaded onto a Hewlett-Packard miniature biphasic reaction column, and washed with 1 ml of 2% trifluoroacetic acid. The sample was sequenced on a Hewlett-Packard Model G1005A protein sequencing system using Edman degradation. Version 3.0 sequencing methods and all reagents were supplied by Hewlett-Packard. Sequence was confirmed for 50 cycles.

Results. Placental Bikunin was purified to apparent homogeniety by sequential kallikrein affinity, gel-filtration, and reverse-phase chromatography (see purification table below):

Table 5
Purification table for native Placental Bikunin (1-179)

TABLE 5

Step	Vol (ml)	OD 280 (/ml)	OD 280	Units[a] (U)	Units/OD 280
Placenta Supernatant	1800.0	41.7	75,060	3,000,000	40.0
Kallikrein Affinity pH 4.0	20.0	0.17	3.36	16,000	4,880
Kallikrein Affinity pH 1.7	10.2	0.45	4.56	12,000	2,630
Superdex 75	15.0	0.0085	0.13	3,191	24,546

[a]One Unit is defined as that amount which inhibits 50% of trypsin activity in a standard assay.

The majority of the kallikrein and trypsin inhibitory activity eluted from the kallikrein affinity column in the pH 4.0 elution. Subsequent gel-filtration chromatography (Figure 5) yielded a peak of kallikrein and trypsin inhibitory

activity with a molecular weight range of 10 to 40 kDa as judged by a standard curve generated by running molecular weight standards under identical conditions. Reverse-phase C18 chromatography (Figure 6) yielded 4 peaks of inhibitory activity with the most potent eluting at approximately 30 % acetonitrile. The activity associated with the first peak to elute from C18 (fraction 29) exhibited an amino acid sequence starting with amino acid 1 of the predicted amino acid sequence of placental bikunin (ADRER...; SEQ ID NO: 1), and was identical to the predicted sequence for 50 cycles of sequencing (underlined amino acids in Figure 3). Cysteine residues within this sequence stretch were silent as expected for sequencing of oxidized protein. The cysteine residues at amino acid positions 11 and 20 of mature placental bikunin were later identified from sequencing of the S-pyridylethylated protein whereupon PTH-pyridylethyl-cysteine was recovered at cycles 11 and 20.

Interestingly, the asparagine at amino acid residue number 30 of the sequence (Figure 3) was silent showing that this site is likely to be glycosylated. Fraction 29 yielded one major sequence corresponding to that of placental bikunin starting at residue #1 (27 pmol at cycle 1) plus a minor sequence (2 pmol) also derived from placental bikunin starting at residue 6 (SIHD...). This shows that the final preparation sequenced in fraction 29 is highly pure, and most likely responsible for the protease inhibitory activity associated with this fraction (Figure 6).

Accordingly, the final preparation of placental bikunin from C18 chromatography was highly pure based on a silver-stained SDS-PAGE analysis (Figure 7), where the protein migrated with an apparent Mr of 24 kDa on a 10 to 20 % acrylamide tricine gel (Novex, San Diego, CA) calibrated with the following molecular weight markers: insulin (2.9 kDa); bovine trypsin inhibitor (5.8 kDa); lysozyme (14.7 kDa); β-lactaglobulin (18.4 kDa); carbonic anhydrase (29 kDa); and ovalbumin (43 kDa). The above size of placental bikunin on SDS-PAGE is consistent with that predicted from the full length coding sequence (Figure 4F).

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-

159) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (Trasylol®).

Table 6
Ki values for the inhibition of various proteases by placental bikunin

TABLE 6

Protease (concentration)	Placental Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.13	0.8
Human Plasmin (50 pM)	1.9	1.3

The results show that placental bikunin isolated from a natural source (human placenta) is a potent inhibitor of trypsin-like serine proteases.

Example 8

Expression pattern of placental bikunin amongst different human organs and tissues

A multiple tissue northern was purchased from Clontech which contained 2 µg of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using ^{32}P-dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

Results. The pattern of tissue expression observed using a placental bikunin (102-159) probe (Figure 11A) or a larger probe containing both Kunitz domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

The broad tissue distribution of the mRNA shows that placental bikunin is broadly expressed. Since the protein also contains a leader sequence it would have ample exposure to the human immune system, requiring that it become recognized as a self protein. Additional evidence for a broad tissue distribution of placental bikunin mRNA expression was derived from the fact that some of the EST entries with homology to placental bikunin (Figure 4B) were derived from human adult and infant brain, and human retina, breast, ovary, olfactory epithelium, and placenta. It is concluded therefore that administration of the native human protein to human patients would be unlikely to elicit an immune response.

Interestingly, the expression pattern of placental bikunin is somewhat reminiscent of that for bovine aprotinin which is found in high levels in bovine lung and pancreas. To further elucidate the expression pattern of placental bikunin, RT-PCR of total RNA from the following human cells was determined: un-stimulated human umbilical vein endothelial cells (HUVECs), HK-2 (line derived from kidney proximal tubule), TF-1 (erythroleukemia line) and phorbolester (PMA)-stimulated human peripheral blood leukocytes. The probes used:

CACCTGATCGCGAGACCCC (sense; SEQ ID NO: 59);
CTGGCGGAAGCAGCGGAGCATGC (antisense; SEQ ID NO: 60),

were designed to amplify a 600 b.p placental bikunin encoding cDNA fragment. Comparisons were normalized by inclusion of actin primers to amplify an 800 b.p. actin fragment. Whereas the 800 b.p fragment identified on agarose gels with ethidium bromide was of equal intensity in all lanes, the 600 b.p. placental bikunin fragment was absent from the HUVECs but present in significant amounts in each of the other cell lines. We conclude that placental

bikunin is not expressed in at least some endothelial cells but is expressed in some leukocyte populations.

Example 9

Purification and properties of Placental Bikunin (1-170) highly purified from a Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector (see previous Examples) was liberated by digestion with HindIII and Xba1 yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a Pst1 site 3' to the XbaI site at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

5' CGC GTC TCG GCT GAC CTG GCC CTG CAG ATG GCG CAC GTG TGC GGG 3' (SEQ ID NO: 61)

A stop codon (TAG) and BglII / XmaI site was similarly engineered at the 3' end of the cDNA using the oligonucleotide:

5' CTG CCC CTT GGC TCA AAG TAG GAA GAT CTT CCC CCC GGG GGG GTG GTT CTG GCG GGG CTG 3' (SEQ ID NO: 62).

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with Pst1 and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) which contains both Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane segment.

The expression of Bikunin by Sf-9 insect cells was optimal at a multiplicity of infection of 1 to 1 when the medium was harvested at 72 h post

infection. After harvesting, the baculovirus cell culture supernatant (2L) was adjusted to pH 8.0 by the addition of Tris-HCl. Bikunin was purified by chromatography using a 5 ml bovine pancreatic kallikrein affinity column as previously described in Example 7 for the purification of native placental bikunin from placenta. Eluted material was adjusted to pH 2.5 with TFA and subjected to chromatography on a C18 reverse-phase column (1.0 x 25 cm) equilibrated in 10% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The bikunin was eluted with a linear gradient of 10 to 80% acetonitrile in 0.1% TFA over 40 min. Active fractions were pooled, lyophilized, redissolved in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 2 mM CaCl2, and 0.1% triton x-100, and stored at -20°C until needed. The concentration of recombinant bikunin was determined by amino acid analysis.

Results. Recombinant bikunin was purified from baculovirus cell culture supernatant using a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 8 below).

Table 8
Purification of recombinant bikunin from transformed culture supernatant

TABLE 8

Purification Step	Vol (ml)	OD 280/ml	OD 280 total	Units (U)	Specific activity (U/OD)
Supernatant	2300.0	9.0	20,700	6,150,000	297
Kallikrein affinity	23.0	0.12	2.76	40,700	14,746
C18 reverse-phase	0.4	3.84	1.54	11,111	72,150

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....) , showing that the signal

peptide was correctly processed in Sf9 cells.

Purified placental bikunin from Sf9 cells (100 pmol) was pyridylethyl-alkylated, CNBr digested and then sequenced without resolution of the resulting fragments. Sequencing for 20 cycles yielded the following N-terminii:

Sequence	Amount	Placental bikunin residue #
LRCFrQQENPP-PLG-----	21 pmol	154 - 168 (SEQ ID NO: 63)
ADRERSIHDFCLVSKVVGRC	20 pmol	1 - 20 (SEQ ID NO: 64)
FNYeEYCTANAVTGPCRASF	16 pmol	100 - 119 (SEQ ID NO: 65)
Pr--Y-V-dGS-Q-F-Y-G	6 pmol	25 - 43 (SEQ ID NO: 66)

Thus N-terminii corresponding to each of the expected four fragments were recovered. This confirms that the Sf9 expressed protein contained the entire ectodomain sequence of placental bikunin (1-170). N-terminal sequencing (50 cycles) of an additional sample of undigested Placental Bikunin (1-170) resulted in an amino acid sequence which at cycle 30 was devoid of any PTH-amino acid (PTH-asparagine was expected). A similar result was obtained upon sequencing of the natural protein from human placenta (Example 7) and is consistent with this residue being glycosylated as predicted from the amino acid sequence surrounding this asparagine residue. Furthermore, the cysteine residues within this region were also silent consistent with their participation in disulfide bonding.

Example 10

Inhibition specificity of purified placental bikunin derived from Sf9 cells.

The *in vitro* specificity of recombinant bikunin was determined using the materials and methods as described in Examples 3, 4 and 7. In addition, the inhibition of human tissue kallikrein by bikunin was measured by the incubation of 0.35 nM human tissue kallikrein recombinant bikunin in buffer containing 50 mM Tris (pH 9.0), 50 mM NaCl, and 0.01% triton x-100. After 5 min. at 37°C, 5 µl of 2 mM PFR-AMC was added and the change in fluorescence monitored.

Inhibition of tissue plasminogen activator (tPA) was also determined as follows: tPA (single chain form from human melanoma cell culture from Sigma Chemical Co, St Louis, MO) was pre-incubated with inhibitor for 2 hr at room temperature in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl, and 0.02% sodium azide. Reactions were subsequently initiated by transfer to a reaction

system comprising the following initial component concentrations: tPA (7.5 nM), inhibitor 0 to 6.6 µM, DIle-Lpro-Larg-pNitroaniline (1mM) in 28 mM Tris buffer pH 8.5 containing 0.004 % (v/v) triton x-100 and 0.005% (v/v) sodium azide. Formation of p-Nitroaniline was determined from the A405nm measured following incubation at 37 C for 2hr.

The table below show the efficacy of recombinant bikunin as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either recombinant bikunin, or aprotinin.

Table 9
Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) or aprotinin

TABLE 9

Protease (concentration)	Recombinant Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.064	0.8
Human Plasma Kallikrein (2.5 nM)	0.18	19.0
Human Tissue Kallikrein (0.35 nM)	0.04	0.004
Bovine Pancreatic Kallikrein (100 pM)	0.12	0.02
Human Plasmin (50 pM)	0.23	1.3
factor Xa (0.87 nM)	180	5% Inhibition at 31 µM
factor XIa (0.1 nM)	3.0	288
tissue plasminogen activator (7.5 nM)	< 60	no inhibition at 6.6 µM
Tissue Factor VIIa	800	no inhibition at 1 µM

The results show that recombinant bikunin can be expressed in insect cells to yield an active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant bikunin was more potent that the synthetically derived bikunin fragments (7-64) and (102-159) against all enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Besides measuring the potencies against specific proteases, the capacity of placental bikunin (1-170) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with

aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra^R 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N.Y.). The instrument was set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl2 were automatically dispensed to initiate clotting, and the clotting time was monitored automatically. The results (Figure 14) showed that a doubling of the clotting time required approximately 2 μM final aprotinin, but only 0.3 μM Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and usefull as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

Although certain embodiments of the invention have been described in detail for the purpose of illustration, it will be readily apparent to those skilled in the art that the methods and formulations described herein may be modified without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WE CLAIM:

1. A substantially purified protein, having serine protease inhibitory activity, selected from the group of proteins consisting of materials each of which comprises one of the following amino acid sequences, the amino acids of said sequences being numbered in accordance with the amino acid sequence of native human placental bikunin shown in figure 4F in which the N-terminal residue generated by removal of signal peptide is designated as residue 1:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK 170
(SEQ ID NO: 52);

MAQLCGL RRSRAFLALL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS SGDDKEQLVK NTYVL 225
(SEQ ID NO: 49);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS SGDDKEQLVK NTYVL 225
(SEQ ID NO: 70);

AGSFLAWL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 2);

MLR AEADGVSRLL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS SGDDKEQLVK NTYVL 225
(SEQ ID NO: 45);

MAQLCGL RRSRAFLALL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 47);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 71);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATV 64
(SEQ ID NO: 4);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK C 61
(SEQ ID NO: 5);

YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6);

CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE 150
ACMLRC 156

(SEQ ID NO: 7);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQ 159
(SEQ ID NO: 3);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRC 156
(SEQ ID NO: 50);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 25
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 1); and

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS 92
(SEQ ID NO: 8).

2. A protein as in claim 1, wherein said protein is glycosylated, or contains at least one intra-chain cysteine-cysteine disulfide bond, or is both glycosylated and contains at least one intra-chain cysteine-cysteine disulfide bond.

3. A pharmaceutical composition for inhibiting serine protease activity, comprising a protein of claim 1 or claim 2 plus a pharmaceutically acceptable carrier.

4. An isolated nucleic acid sequence which encodes for a protein of claim 1.

5. A self-replicating protein expression vector containing a nucleic acid sequence which encodes for and is capable of expressing a protein of claim or claim 2.

6. A method for inhibiting serine protease activity comprising contacting serine protease with an effective amount of at least one protein of claim 1 or claim 2.

7. A method for treating a condition of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, polytrauma, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis comprising administering to a subject having such a condition an effective amount of the protein of claim 1 or claim 2.

8. The method of Claim 7 wherein said condition is brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, or open heart surgery.

9. The method of Claim 7 wherein said condition is gastric cancer, cervical cancer, or prevention of metastasis.

10. A method for the preparation of a medicament for the treatment of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis.

11. A method for preparing a protien of claim 1 or claim 2 using recombinant DNA technology.

FIGURE 1

```
R35464 GGCCGGGTCG TTTCTCGCCT GGCTGGGATC GCTGCTCCTC TCTGGGGTCC   50
ORF      P  G  R  F  S  P  G  W  D  R  C  S  S  L  G  S      16

R35464 TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  100
ORF    W  P  A  D  R  E  R  S  I  H  D  F  C  L  V  S  K     33

R35464 GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA  150
ORF    V  V  G  R  E  R  A  S  M  P  R  W  W  Y  N  V  T     50

R35464 CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC  200
ORF      D  G  S  C  Q  L  F  V  Y  G  G  C  D  G  N  S      66

R35464 AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  250
ORF    N  N  Y  L  T  K  E  E  C  L  K  K  C  A  T  V  T     83

R35464 AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT  300
ORF      E  N  A  T  G  D  L  A  T  S  R  N  A  A  D  S  S  100

R35464 CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA  350
ORF      V  P  S  A  P  R  R  Q  D  S  *  R  P  L  Q  R     116

R35464 TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT-------  393
ORF    Y  V  S  *  I  *  R  I  I  A  P  *  T  *              130
```

KEY
R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO: 12)
ORF = EST R35464 Open Reading Frame Translation (SEQ ID NO: 13)

FIGURE 2

```
R74593 GCAATAATTA CCTGACCAAG GAGGAGTGCC TCAAGAAATG TGCCACTGTC   50
ORF      Q  *  L   P  D  Q  G   G  V  P   Q  E  M   C  H  C  H   17

R74593 ACAGAGAATG CCACGGGTGA CCTGGCCACC AGCAGGAATG CAGCGGATTC  100
ORF      R  E  C   H  G  *   P  G  H  Q   Q  E  C   S  G  F    33

R74593 CTCTGTCCCA AGTCTCCCAG AAGGCAGGAT TCTGAAGACC ACTCCAGCGA  150
ORF    L  C  P  K   S  P  R   R  Q  D   S  E  D  H   S  S  D   50

R74593 TATGTTCAAC TATGAAGAAT ACTGCACCGC CAACGCAGTC ACTGGGCCTT  200
ORF      M  F  N   Y  E  E  Y   C  T  A   N  A  V   T  G  P  C   67

R74593 GCCGTGCATC CTTCCCACGC TGGTACTTTG ACGTGGAGAG GAACTCCTGC  250
ORF      R  A  S   F  P  R   W  Y  F  D   V  E  R   N  S  C    83

R74593 AATAACTTCA TCTATGGAGG CTGCCGGGGC AATAAGAACA GCTACCGCTC  300
ORF    N  N  F  I   Y  G  G   C  R  G   N  K  N  S   Y  R  S  100

R74593 TGAGGAGGCC TGCATGCTCC GCTGCTTCCG CCAGCAGGAG AATCCTCCCC  350
ORF      E  E  A   C  M  L  R   C  F  R   Q  Q  E   N  P  P  L  117

R74593 TGCCCCTTGG CTCAAAGGTG GTGGTTCTGG CCGGGGCTGT TTCGTGATGG  400
ORF      P  L  G   S  K  V   V  V  L  A   G  A  V   S  *  W   133

R74593 TGTTGATCCT TTTCCTGGGG AGCNTCCATG GTCTTACTGA TTCCGGGTGG  450
ORF    C  *  S  F   S  W  G   A  S  M   V  L  L  I   P  G  G  150

R74593 CAAGGAGGAA CCAGGAGCGT GCCCTGCGGA NCGTCTGGAG CTTCGGAGAT  500
ORF      K  E  E   P  G  A  C   P  A  X   R  L  E   L  R  R  *  167

R74593 GACAAGGGNT                                             510
ORF      Q  G                                                 169
```

KEY
R74593 = Nucleic acid sequence of EST R74593 (SEQ ID NO: 14)
ORF = EST R74593 Open Reading Frame Translation (SEQ ID NO: 15)

FIGURE 3

```
R35464          GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
N39798                                 TGGGANTC GCTGCTCCTC TCTGGGGTCC  28
H94519           GCNGCG-CGT TNNTCGCNT- GCTGGGA-TC GCTGCACCTC TCTGGGGTCG  47
R74593 corr.     ---------- ---------- ---------- ---------- ----------
Consensus       GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
Translation      A  G  S  F   L  A  W   L  G   S   L  L  L   S  G  V    -3

R35464           TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA 100
N39798           TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  77
H94519           NGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  96
R74593 corr.     ---------- ---------- ---------- ---------- ----------
Consensus        TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  99
Translation      L  A  A  D   R  E  R   S  I  H   D  F  C  L   V  S  K   15

R35464           GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 150
N39798           GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 127
H94519           GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 146
R74593 corr.     ---------- ---------- ---------- ---------- ----------
Consensus        GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 149
Translation      V  V  G   R  C  R  A   S  M  P   R  W  W   Y  N  V  T  32

R35464           CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 200
N39798           CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 177
H94519           CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 196
R74593 corr.     ---------- ---------- ---------- ---------- --------GC   2
Consensus        CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 199
Translation        D  G  S   C  Q  L   E  V  X  G   G  C  D   G  N  S   48

R35464           AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 250
N39798           AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 227
H94519           AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 246
R74593 corr.     AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  52
Consensus        AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 249
Translation      N  N  Y  L   T  K  E   E  C  L   K  K  C  A   T  V  T   65

R35464           AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 300
N39798           AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 277
H94519           AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 296
R74593 corr.     AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 102
Consensus        AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 299
Translation      E  N  A   T  G  D  L   A  T  S   R  N  A   A  D  S  S   82

R35464           CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA 350
N39798           CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 326
H94519           CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 345
R74593 corr.     CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 151
Consensus        CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 348
Translation        V  P  S   A  P  R   R  Q  D  S    E  D  H   S  S  D   98

R35464           TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT-------- 393
N39798           TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 372
H94519           TATGTT-CAA CTA-TG-AAG AATACTGGCA CCGCCAACGC ATTCACTGGG 392
R74593 corr.     TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 197
Consensus        TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 394
Translation      M  F   N   Y   E   E   Y  C   T   A  N   A   V  T  G  113
```

FIGURE 3 (CON'T)

```
R35464          ---------- ---------- ---------- ---------- ----------
N39798          CCTTGC-GTG GAATCCTTTC CCACGCTGGN AATTTNGACG TTGAGAAGGA 421
H94519          CCT-GC-GTG -CATCCTT-C CCACGCTGGT ACTTT-GNCG ---------- 427
R74593 corr.    CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 243
Consensus       CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 440
Translation     P  C  R  A    S  F    P  R  W  Y   F   D  V   E  R   N 129

R35464          ---------- ---------- ---------- ---------- ----------
N39798          AC-------- ---------- ---------- ---------- ---------- 423
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 293
Consensus       ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 490
Translation       S  C  N   N  F  I   Y  G  G  C   R  G  N   K  N  S   145

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 343
Consensus       TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 540
Translation     Y  R  S  E   E  A  C   M  L  R   C  F  R  Q   Q  E  N 162

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 393
Consensus       TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 590
Translation      P  P  L   P  L  G  S   K  V  V   V  L  A   G  A  V  S 179

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 443
Consensus       CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 640
Translation       *  W  C   *  S  F   S  W  G  A   S  M  V   L  L  I   195

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 493
Consensus       CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 690
Translation     P  G  G  K   E  E  P   G  A  C   P  A  *  R   L  E  L 212

R35464          ---------- --------
N39798          ---------- --------
H94519          ---------- --------
R74593 corr.    TCGGAGATGA CAAGGGNT                                    511
Consensus       TCGGAGATGA CAAGGGNT                                    708
Translation      R  R  *   Q  G                                        217
```

KEY

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO.: 12)
N39798 = Nucleic acid sequence of EST N39798 (SEQ ID NO.: 17)
H94519 = Nucleic acid sequence of EST H94519 (SEQ ID NO.: 16)
R74593 corr. = Corrected version of (SEQ ID NO.: 14) G at b.p. 114
Consensus = Nucelic acid sequence for human bikunin (SEQ ID NO.: 9)
Translation = Amino acid Translation of Consensus (SEQ ID NO.: 10)

Figure 4 A.

Schematic depicting the overlap of ESTs bearing homology to the cDNA sequence encoding placental bikunin

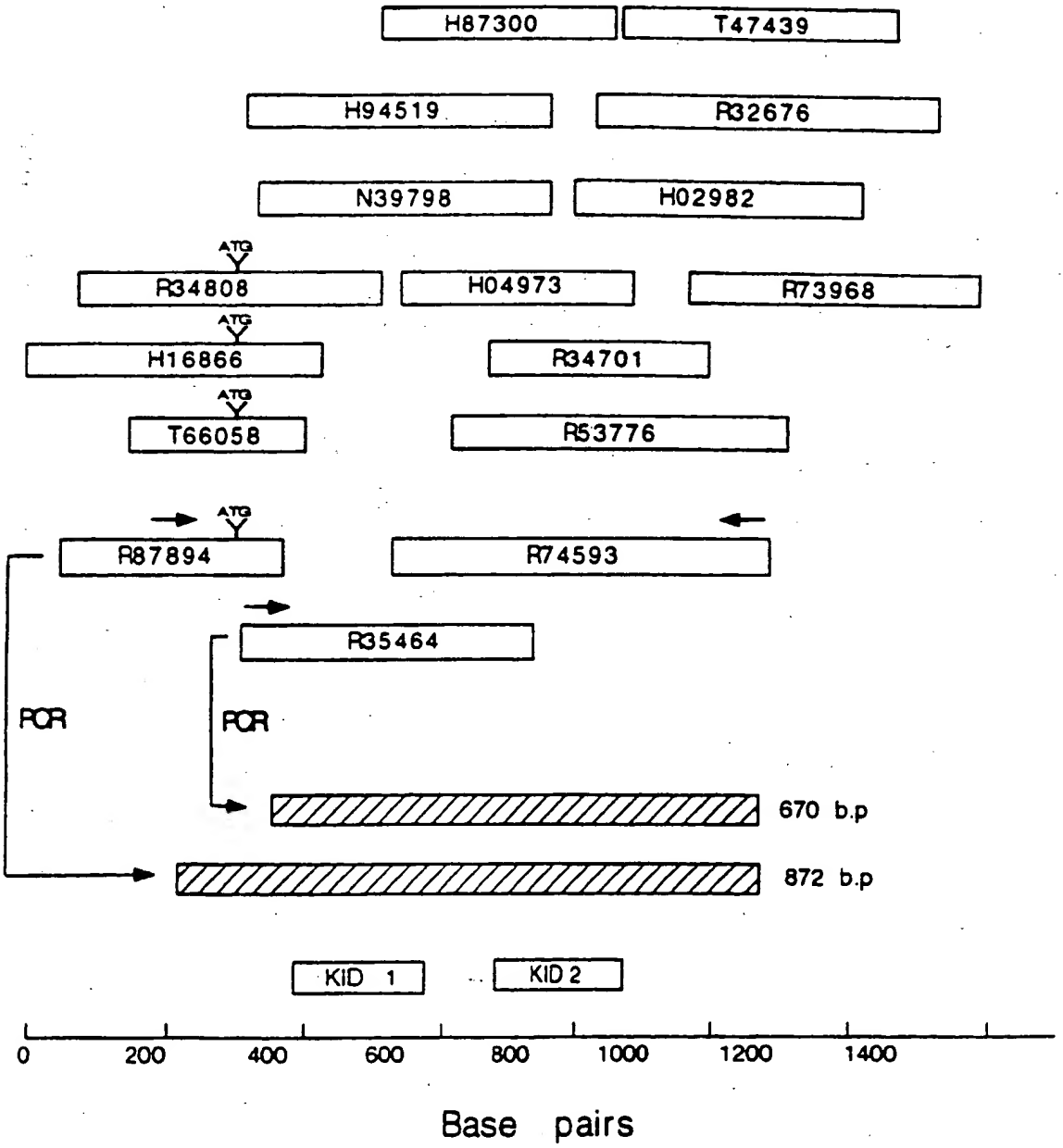

Figure 4B

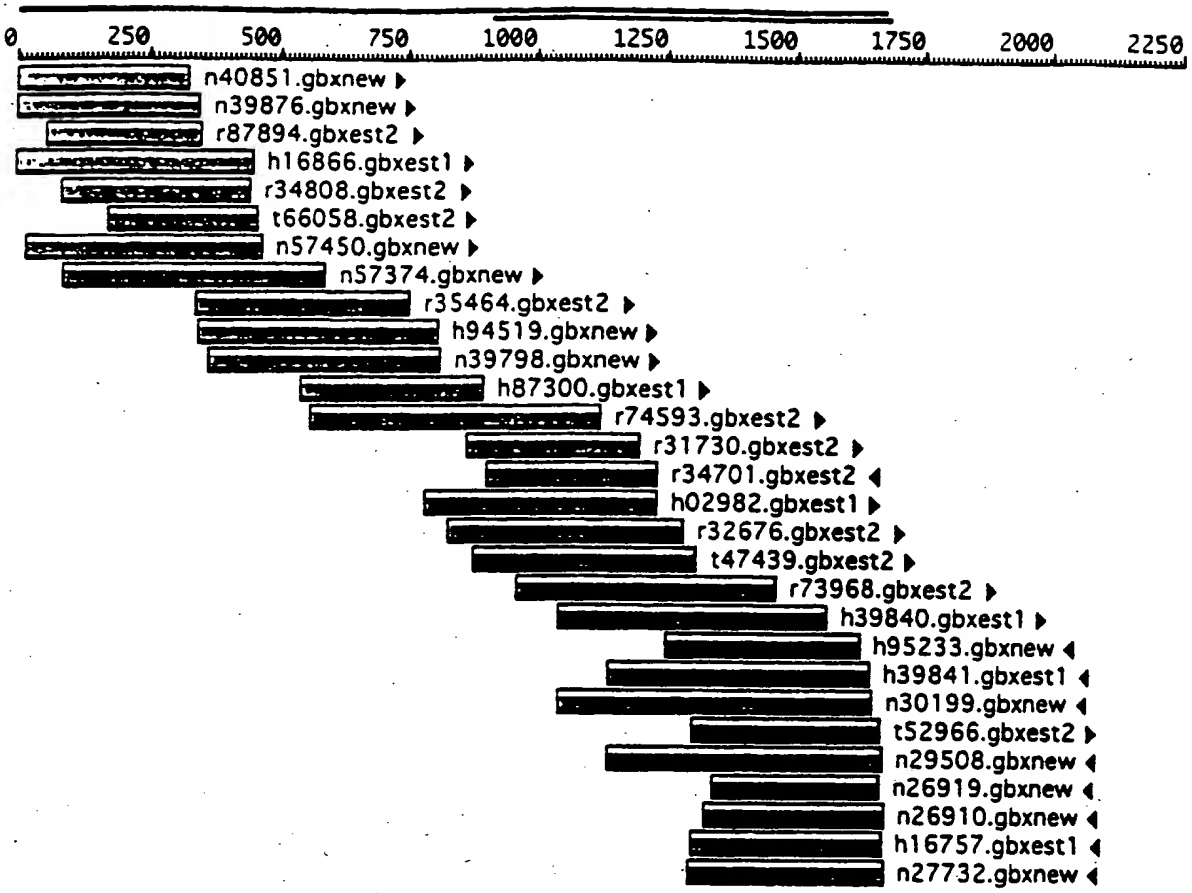
0
250
500
750
1000
1250
1500
1750
2000
2250
n40851.gbxnew
n39876.gbxnew
r87894.gbxest2
h16866.gbxest1
r34808.gbxest2
t66058.gbxest2
n57450.gbxnew
n57374.gbxnew
r35464.gbxest2
h94519.gbxnew
n39798.gbxnew
h87300.gbxest1
r74593.gbxest2
r31730.gbxest2
r34701.gbxest2
h02982.gbxest1
r32676.gbxest2
t47439.gbxest2
r73968.gbxest2
h39840.gbxest1
h95233.gbxnew
h39841.gbxest1
n30199.gbxnew
t52966.gbxest2
n29508.gbxnew
n26919.gbxnew
n26910.gbxnew
h16757.gbxest1
n27732.gbxnew

Figure 4C

```
          1                                                  50
Bikunin   ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N40851    ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N39876    ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
R87894    .......... .......... .......... .......... ..........
H16866    .....GGCGA CCTCCGCGCG TTGGGAGGTG TAGCGCG.CT CTGAACGGGN
R34808    .......... .......... .......... .......... ..........
T66058    .......... .......... .......... .......... ..........
N57450    .......... .......... .........T TAGCGCGGCT CTGAACGCNA
N57374    .......... .......... .......... .......... ..........
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          51                                                        100
Bikunin   GNA GGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N40851    NGAGNGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N39876    GCA.GGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
R87894    .......... TTGAGTGTNG NAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
H16866    ..ANGGGCCG TTGAGTGTCG CAGGCGGC.A GGGCN.GAGT GAGGAGCAGA
R34808    .......... .......... .......... .........G GAGGAGCAGA
T66058    .......... .......... .......... .......... ..........
N57450    GAAGNGGCCG TTGAGTGTCG CAGGCGGCGA GGGCGCGAGT GAGGAGCAGA
N57374    .......... .......... .......... .......... .......AGA
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          101                                                       150
Bikunin   CCCAGGCATC GCGCGCCGAG AAGNC GGGC GTCCCCACAC TGAAGGTCCG
 N40851   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 N39876   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC NTCCCCACAC TGAAGGTCCG
 R87894   CCCAGGCATC GCGCGCCGAG AAGGCCGGGC GTCCCCACAC TGAAGGTCCG
 H16866   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 R34808   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 T66058   .......... .......... .......... .......... ..........
 N57450   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 N57374   CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 R35464   .......... .......... .......... .......... ..........
 H94519   .......... .......... .......... .......... ..........
 N39798   .......... .......... .......... .......... ..........
 H87300   .......... .......... .......... .......... ..........
 R74593   .......... .......... .......... .......... ..........
 R31730   .......... .......... .......... .......... ..........
 R34701   .......... .......... .......... .......... ..........
 H02982   .......... .......... .......... .......... ..........
 R32676   .......... .......... .......... .......... ..........
 T47439   .......... .......... .......... .......... ..........
 R73968   .......... .......... .......... .......... ..........
 H39840   .......... .......... .......... .......... ..........
 H95233   .......... .......... .......... .......... ..........
 H39841   .......... .......... .......... .......... ..........
 N30199   .......... .......... .......... .......... ..........
 T52966   .......... .......... .......... .......... ..........
 N29508   .......... .......... .......... .......... ..........
 N26919   .......... .......... .......... .......... ..........
 N26910   .......... .......... .......... .......... ..........
 H16757   .......... .......... .......... .......... ..........
 N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          151                                                        200
Bikunin   GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
N40851    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
N39876    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
R87894    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
H16866    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACG.T CCCGGAGCN.
R34808    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
T66058    .......... .......... .......... ...GGACCCT CCCGGAGCGT
N57450    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
N57374    GAAAGGCGAC TTCCGGGGGC TTTGGCACCT GGCGGACCCT CCCGGAGCGT
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (C n't)

```
          201                                                       250
Bikunin   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
 N40851   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG .AGGGGCTTC
 N39876   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
 R87894   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
 H16866   .GGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
 R34808   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG GAGGGGCTTC
 T66058   CGGCACCTGA ACGCGAGGC. CTCCATTGCG .GTGCGTGTG NAGGGGCTTC
 N57450   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
 N57374   CGGCACCTGA ACGCGAGGC. CTCCATTGC. CGTGCGTTNG .AGGGGCTTC
 R35464   .......... .......... .......... .......... ..........
 H94519   .......... .......... .......... .......... ..........
 N39798   .......... .......... .......... .......... ..........
 H87300   .......... .......... .......... .......... ..........
 R74593   .......... .......... .......... .......... ..........
 R31730   .......... .......... .......... .......... ..........
 R34701   .......... .......... .......... .......... ..........
 H02982   .......... .......... .......... .......... ..........
 R32676   .......... .......... .......... .......... ..........
 T47439   .......... .......... .......... .......... ..........
 R73968   .......... .......... .......... .......... ..........
 H39840   .......... .......... .......... .......... ..........
 H95233   .......... .......... .......... .......... ..........
 H39841   .......... .......... .......... .......... ..........
 N30199   .......... .......... .......... .......... ..........
 T52966   .......... .......... .......... .......... ..........
 N29508   .......... .......... .......... .......... ..........
 N26919   .......... .......... .......... .......... ..........
 N26910   .......... .......... .......... .......... ..........
 H16757   .......... .......... .......... .......... ..........
 N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          251                                                       300
Bikunin   CCGCACCT G ATCGCGAGAC CCCAACGGCT GGTGG CGTC GC TG CGCG
N40851    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
N39876    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
R87894    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TN.CGCG
H16866    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TGGCGCG
R34808    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGGGCGTC GC.TG.CGCG
T66058    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
N57450    CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
N57374    CCGGAACTTG ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
R35464    .......... .......... .......... .......... ..........
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
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Figure 4C (C n't)

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          301                                                                 350
Bikunin   TC TCGGCTG AGCT GGCCA TGGCGCANT  GTTGC GGGC T GAGGC GG
N40851    TC.TCGGCTG AGCT.GGNCA TGTCG
N39876    TC.TCGGCTG AGCT.GGCCA TGGCGCACT. G.TGCGGNGC T.GAGGC.G
R87894    TC.TCGGCTG AGCTTGGCCA TGGCGCANT. GTTNC.GGGC T.NAGGC.GG
H16866    TTCTCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
R34808    TCTTCGGCTG AGCTGGGCCA TGGCGCANTT GTTGC.GGGC T.GAGGC.GG
T66058    TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
N57450    TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GGTGC.GGGC TTGAGGC.GG
N57374    TCCTCGGCTG AGCT.GGCCA TGGCGCANT. GGTGCCGNGC T.GAGGCCGG
R35464    .......... .......... .......... .......... ....GGCCGG
H94519    .......... .......... .......... .......... ..........
N39798    .......... .......... .......... .......... ..........
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          351                                                        400
Bikunin   AC  GG CG    TTTCTCG  CC TGCTGGG A TCGCT GC T CCTCTCT
R87894    ACG.
H16866    AC..CGNCGT TTTTCTTCG. CCTTGCTGGG ATTCGCTTGC TTCCTNTCTG
R34808    ACGCGGNCG. .TTTTTTCGN CCTTGCTGGG ATTCG.TTG. TTNCTCTCTN
T66058    ...CGGNCG. .TTTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57450    ANN.NGCCG. ..TTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57374    AG..GGCCGG ..TTTCTCG. CCTTGCTGGG A.TCGCT.GC T.CCTCTCTG
R35464    .....GTCG. ..TTTCTCG. CCTGGCTGGG A.TCGCT.GC T.CCTCTCT.
H94519    .GCNGCGCG. ..TTNNTCG. CN.TGCTGGG A.TCGCT.GC A.CCTCTCT.
N39798    .......... .......... .....CTGGG ANTCGCT.GC T.CCTCTCT.
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          401                                                       450
Bikunin   GGGG TCCTG G  CGGCCGA CCGA GAACG CA GCA TCC ACGACTT CT
H16866    GGGGTTCCTG GG.CGGCCGA CCGA.GAACG CA.GCA.TCC AAGAATTTTT
R34808    GGGGTTC.TG GGGNGGCCGA NCGA.GAACG CAAGCA.TTC ACGA.TTT
T66058    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGANTT.CT
N57450    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N57374    GGGG.TCCTG G..CGGCCGA NCGAAGAANG CA.GCAATCC ANGAATTNCT
R35464    GGGG.TCCTG G.CCGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H94519    GGGG.TCGNG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N39798    GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          451                                                        500
Bikunin   GCCTGGTGT  CGAAGGT GG TGGGCAGATG CCGGG CCTC CATGCCTA G
 H16866   GCC
 T66058   TCCTGGTGTT CGAAGG
 N57450   GCCTGGTGT. CGAAGGT.GG TGGGCAG
 N57374   GCCTGGTGTT CGAAAGTTGG TGGGCANATT CCGGGGCCTT CATGNCTAAG
 R35464   GCCTGGTGT. CGAAGGT.GG TGGGCAGATT CCGGG.CCTC CATGCCTA.G
 H94519   GCCTGGTGT. CGAAGGT.GG TGGGCAGATG CCGGG.CCTC CATGCCTA.G
 N39798   GCCTGGTGT. CGAAGGT.GG TGGGCAGATG CCGGG.CCTC CATGCCTA.G
 H87300   .......... .......... .......... .......... ..........
 R74593   .......... .......... .......... .......... ..........
 R31730   .......... .......... .......... .......... ..........
 R34701   .......... .......... .......... .......... ..........
 H02982   .......... .......... .......... .......... ..........
 R32676   .......... .......... .......... .......... ..........
 T47439   .......... .......... .......... .......... ..........
 R73968   .......... .......... .......... .......... ..........
 H39840   .......... .......... .......... .......... ..........
 H95233   .......... .......... .......... .......... ..........
 H39841   .......... .......... .......... .......... ..........
 N30199   .......... .......... .......... .......... ..........
 T52966   .......... .......... .......... .......... ..........
 N29508   .......... .......... .......... .......... ..........
 N26919   .......... .......... .......... .......... ..........
 N26910   .......... .......... .......... .......... ..........
 H16757   .......... .......... .......... .......... ..........
 N27732   .......... .......... .......... .......... ..........
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Figure 4C (Con't)

```
          501                                                        550
Bikunin   G TGGT GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
N57374    GTTGGTTGGT ANAATGTNAA TTAANGATTC TTGCAACTGT TTGTGTNATT
R35464    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
H94519    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
N39798    G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
H87300    .......... .......... .......... .......... ..........
R74593    .......... .......... .......... .......... ..........
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          551                                                        600
Bikunin   GGGGCTGTGA   CGGAAACA GCAATAATTA CCTGACCAAG GA GGAGTGC
N57374    GGGGCTNTTA AACGGAAANA .CAATAATNA CCTGACCAAA GAAGNAAT..
R35464    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H94519    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
N39798    GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H87300    GATTCGGCAC AGGGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTNC
R74593    .......... .......... GCAATAATTA CCTGACCAAG GA.GGAGTGC
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          601                                                       650
Bikunin   CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R35464    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H94519    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
N39798    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H87300    CTCAAGAAAT GTNCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R74593    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          651                                                       700
Bikunin   CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R35464    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H94519    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
N39798    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H87300    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R74593    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGT.CTCCC AGAAGGCAGG
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          701                                                       750
Bikunin   ATTCT GAAG ACCACTCCAG CGATATGTT  CAACTAT  G AAGAATACTG
R35464    ATTCTTGAAG ACCACTTCAG CGATATGTTT CAANTATTGN AAGAATAATT
H94519    ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
N39798    ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
H87300    ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R74593    ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R31730    ..... ..... ..... ..... .....
R34701    ..... ..... ..... ..... .....
H02982    ..... ..... ..... ..... .....
R32676    ..... ..... ..... ..... .....
T47439    ..... ..... ..... ..... .....
R73968    ..... ..... ..... ..... .....
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....

          751                                                       800
Bikunin    CACCGCCAA CGCAGT CAC TGGGCC TTG CCGTG CAT  CCTT CCCAC
R35464    GCACCGNCAA CGNATT
H94519    GCACCGCCAA CGCATT.CAC TGGGCC..TG C.GTG.CAT. CCTT.CCCAC
N39798    .CACCGCCAA CGCAGT.CAC TGGGGCCTTG C.GTGGAAT. CCTTTCCCAC
H87300    .CACCGCCAA CGCAGTNCAC TGGGCC.TTG C.GTGGCATN CCTT.CCCAC
R74593    .CACCGCCAA CGCAGT.CAC TGGGCC.TTG CCGTG.CAT. CCTT.CCCAC
R31730    ..... ..... ..... ..... .....
R34701    ..... ..... ..... ..... .....
H02982    ..... ..... ..... ..... .....
R32676    ..... ..... ..... ..... .....
T47439    ..... ..... ..... ..... .....
R73968    ..... ..... ..... ..... .....
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....
```

Figure 4C (Con't)

```
          801                                                       850
Bikunin   GCTGGTACTT T GACGTGGA GA GGAACTC CTG CAATAA CTTCATCTAT
 H94519   GCTGGTACTT T.GNCGT
 N39798   GCTGGNAATT TNGACGTTGA GAAGGAAC
 H87300   GCTNGTACTT T.GACGTGGA GA.GGAACTC CTGGCAATAA CTTCATCTAT
 R74593   GCTGGTACTT T.GACGTGGA GA.GGAACTC CTG.CAATAA CTTCATCTAT
 R31730   .......... .......... .......... .......... ..........
 R34701   .......... .......... .......... .......... ..........
 H02982   .......... ........GA GA.GGAACTC CTG.CAATAA CTTCATCTAT
 R32676   .......... .......... .......... .........G ATTC..GGAA
 T47439   .......... .......... .......... .......... ..........
 R73968   .......... .......... .......... .......... ..........
 H39840   .......... .......... .......... .......... ..........
 H95233   .......... .......... .......... .......... ..........
 H39841   .......... .......... .......... .......... ..........
 N30199   .......... .......... .......... .......... ..........
 T52966   .......... .......... .......... .......... ..........
 N29508   .......... .......... .......... .......... ..........
 N26919   .......... .......... .......... .......... ..........
 N26910   .......... .......... .......... .......... ..........
 H16757   .......... .......... .......... .......... ..........
 N27732   .......... .......... .......... .......... ..........

          851                                                       900
Bikunin   GGAGGCT GC CGGGGCAAT  AAGAACAG C TACCGCTC T GAGGAGGCCT
 H87300   GGAGGCTTGC CGGGGCAATN AAGAACAGNT TACCGCTCTT TAGGAGGCCT
 R74593   GGAGGCT.GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT
 R31730   .......... .......... .......G.C TACCGCTC.T GAGGAGGCCT
 R34701   .......... .......... .......... .......... ..........
 H02982   GGNGGCT.GC CGGGG.AAT. AAGAACA.NC TACCGCTC.T GAGGAGGCCT
 R32676   CGAGGA..GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT
 T47439   .......... .......... .......... .......... ....NGGCCT
 R73968   .......... .......... .......... .......... ..........
 H39840   .......... .......... .......... .......... ..........
 H95233   .......... .......... .......... .......... ..........
 H39841   .......... .......... .......... .......... ..........
 N30199   .......... .......... .......... .......... ..........
 T52966   .......... .......... .......... .......... ..........
 N29508   .......... .......... .......... .......... ..........
 N26919   .......... .......... .......... .......... ..........
 N26910   .......... .......... .......... .......... ..........
 H16757   .......... .......... .......... .......... ..........
 N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          901                                                                 950
Bikunin   GCA TGCTC CGCTGCTTCC GC                                        CA GCAGGA
H87300    .GCA.T.... .
R74593    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R31730    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R34701    ..... .....TTCC GC..... ..... .CAAGCAGGA
H02982    .GCG.TGCTC CGCTGCTTCC GCTGTGTGTT CTCTTCCAGG CCA.GCAGGA
R32676    .GCA.TGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
T47439    TGCAGTGCTC CGCTGCTTCC GC..... ..... .CA.GCAGGA
R73968    ..... ..... ..... ..... .....
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....

          951                                                                1000
Bikunin   GAA TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC  TGG CGGGGC
R74593    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGGCCGGGGC
R31730    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R34701    AAANTCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTCC TGG.CGGGGC
H02982    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R32676    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
T47439    GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R73968    ..... ..... ..... ..... ....CGGGGC
H39840    ..... ..... ..... ..... .....
H95233    ..... ..... ..... ..... .....
H39841    ..... ..... ..... ..... .....
N30199    ..... ..... ..... ..... .....
T52966    ..... ..... ..... ..... .....
N29508    ..... ..... ..... ..... .....
N26919    ..... ..... ..... ..... .....
N26910    ..... ..... ..... ..... .....
H16757    ..... ..... ..... ..... .....
N27732    ..... ..... ..... ..... .....
```

Figure 4C (Con't)

```
          1001                                                      1050
Bikunin   TGTT CGTGA TGGTGTTGAT CC T CTTCC TGGG AGCCT CC ATGGTC
R74593    TGTTTCGTGA TGGTGTTGAT CCTT..TTCC TGGGGAGCNT CC.ATGGTCT
R31730    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGGGAGCCT CC.ATGGTC.
R34701    TGTT.CGTGA TGGTGTTGAT CCCTCCTTCC CGGG.AGCCT CCCATGGTCC
H02982    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTN.
R32676    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
T47439    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
R73968    TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1051                                                      1100
Bikunin   TACC TGAT  CCGGGTGGCA CGGAGG AAC C AGG AGCG TGCCCTGCGC
R74593    TAC..TGATT CCGGGTGGCA AGGAGG.AAC C.AGG.AGCG TGCCCTGCGG
R31730    TACC.TGAT. CCGGGTGGCA CGGAGGGAAC C.AGGGAGCG TGCCCTGCGC
R34701    TACCCTGAT. CCGGGTGGCA CGGAGG.AAC CCAGG.ANCG TGCCCTGCGC
H02982    TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGN
R32676    TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGC
T47439    TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
R73968    TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
H39840    .......... .......... ...GGG.AAC C.AGG.AGCG TGCCCTGCGC
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... ..GAGGAACC C.ANG.AGCT TCCCCTGCGC
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          1101                                                                1150
Bikunin   ACCG TCT G GAGCTCCGGA GATGACAAGG  AGCAGCTGG  TGAAGAAC
R74593    ANCG.TCT.G GAGCTTCGGA GATGACAAGG GNT
R31730    ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG GTGAAGAAC.
R34701    ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H02982    ACCG.TCTNG GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
R32676    ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG .TGAAGAAC.
T47439    ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
R73968    ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H39840    ACCGGTCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    ACCG.TCT.G GAGCTCCGGA GATNACAANG .AGCAGCTGN .TGAAGAACC
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1151                                                                1200
Bikunin   ACATATGT C CTGT GACCG CCCTGT CGC C AAGAGG A CT GGGGAA
R31730    ACATATGTTC CTGTTGACCG NCCTGTTCGC C.AAGAGG.A TTGGGGGAA.
R34701    ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
H02982    ACATATGT.C CTGT.GACCG NCCTGTTCGN C.AAGAGG.A CT.GGGGAA.
R32676    ACATATGTTC CTGTTGACCG CCCTGTTCGC C.AAGAGGGA CTNGGGGAAA
T47439    ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGGGA NTGGGGGAA.
R73968    ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
H39840    ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
H95233    .......... .......... .......... .......... ..........
H39841    .......... ........C. CCCTGT.CGC CCAAAAGG.A CT.GGGGAA.
N30199    ACATATGT.C CTGT.GACCG CCCTNT.CGC C.AAGAGG.A CT.GGGNAAA
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......CC. CCCTNT.CGC C.AAGAGG.A CT.GGG.AA.
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          1201                                                      1250
Bikunin   GGGAGGGG AGACTAT G TGT GA GCT TTTTTT AA A TAGA GG
R31730    .GGGAGGGGG A
R34701    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TA
H02982    GGGGAGGGG. AGATTAT.G. TGTTGA.GTT TTTTTT..AA ANTAG
R32676    GGGGAGGGGG AGANTATTGT TGTTGA.GNT TTTTTTTAAA ATTAGGAGGG
T47439    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
R73968    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
H39840    .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
H95233    .......... .......... .......... .......... ..........
H39841    .GGGAGGGGA AAACNAT.G. TGT.GAACCT TTTTTT.AAA A.TAGA..GG
N30199    .GGGAGGNG. AGACTAT.G. TGT.AA.GCT TTTTTT..AA A.TAGA..GG
T52966    .......... .......... .......... .......... ..........
N29508    .GGGAGGGG. AGACTA..G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1251                                                      1300
Bikunin   GATTGACTC  GGATTTG A GT GATC A  TTAGGG  CT GAGGTCTGTT
R32676    GNTTGANTTC GGGNTTTTNA GTTGATCCAT TTAGGGGGNT GAG
T47439    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTNTT
R73968    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
H39840    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
H95233    .......... .......... ........A. TTAGGG..CT GAGGTCTGTT
H39841    GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
N30199    GATTGACTC. .GGATTTGGA GT.GATC.A. TTAGGG..CT GAGGTCTGTT
T52966    .......... .......... .......... .......... ..........
N29508    GATTGACTC. .GGATTTG.A GT.GATCNA. TTAGGG..CT GAGGTCTGTT
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          1301                                                      1350
Bikunin   TCTCTGGGAG GTAGGACGGC TGCTTCC TG G TC TGGCA  GGGATGGG
T47439    TCTCTNGGAG GTAGGACGA
R73968    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG GGTCTTGGCA .GGGATGGGG
H39840    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
H95233    NCTCTGGGAG NTAGGACGGC TGCCTTCCTG G.TC.TGGCA .GGGATGGG.
H39841    TCNCTGGGAG GTAGGACGGC TGCTCCCCTG G.TC.TGGCA .GGGATGGG.
N30199    TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
T52966    .......... .......... .......... ..TC.TGGCA .GGGATGGG.
N29508    TCTCTGGGAG GTAGGACGGC TGCTTCA.TG G.TC.TGGCA .GGGATGGG.
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... ...T.....G G.TC.TGGCA .GGGATGGG.
N27732    .......... .......... .....CCCTG GGTCCTGNCA AGGNATGGGG
```

Figure 4C (Con't)

```
          1351                                                    1400
Bikunin   TTTG CTTTG G AAATCCTC T AGGAGGCT CCTCCT CGC ATGG CC TG
R73968    TTTG.CTTTG GGAAATCCTC TTNGGAGGCT CCTCCTTCGC ATGGGCCTTG
H39840    TTTG.CTTTG GAGAATCCTC T.ANGAGGCT CCTCCT.CGC ATGG.CC.TG
H95233    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H39841    TTTG.CTTTG G.AAANCCNC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N30199    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCTTCGC ATGG.CC.TG
T52966    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N29508    TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N26919    .......... .......... ....GAGGCT CCTCCT.CGC ATGG.CC.TG
N26910    .....CTTTT GNAAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H16757    TTTGCCTTTG G.AAANCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N27732    TTTG.CTTTG G.AAATCCTC TTAGGAGGCT CCTCCT.CGC ATGG.CC.TG

          1401                                                    1450
Bikunin   CAGT CT GG CAGCAG CCC CGAGTTGTTT  CC TCGCTG ATC GATTTC
R73968    CAGT.CTNGG CAGCANCCCC CGAGTTTTTT TCCTTCGCTG ATCCGATTTC
H39840    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H95233    CAGTTCT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H39841    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTN .CC.TCGCTG ATC.GATNTC
N30199    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
T52966    CAGT.CT.GG CAGCAG..CC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N29508    CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N26919    CAGT.CTTGG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC
N26910    CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATCGGATTTC
H16757    CAGTNCT.GG CAGCAGACCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N27732    CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC

          1451                                                    1500
Bikunin   TTT CCTCCA GGTAG  AGT TTTC TTTG  CTTATGTTGA ATTCCATTGC
R73968    TTTTCCTCCA GGTAAGAATT TTTCTTTT
H39840    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H95233    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H39841    TTT.CCCCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ANTCCATTGC
N30199    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
T52966    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N29508    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26919    TTT.CCNCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26910    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H16757    TTTACCCCCA GGTAG..AGT TTTCCTTTGN CTTATGTTGA ATTCCATTGC
N27732    TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
```

Figure 4C (Con't)

```
          1501                                                    1550
Bikunin   CTCTTTT CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT GT
H39840    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTTTGT
H95233    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
H39841    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N30199    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
T52966    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N29508    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N26919    CTCTTTT.CN CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N26910    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
H16757    CTCTTTTACT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
N27732    CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT

          1551                                                    1600
Bikunin   CTGATTTATG G  TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H39840    CTGATTTATG GGTTTTTTTT AAGTAT
H95233    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H39841    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N30199    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
T52966    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N29508    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N26919    CTGATTTATG G..TTTTTTT AAGTNTAAAC AAAAGTTTTT TATTAGCATT
N26910    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
H16757    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
N27732    CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT

          1601                                                    1650
Bikunin   CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
H95233    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAA
H39841    CTGAAAGAAG GAAAGTAAAN TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N30199    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
T52966    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N29508    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N26919    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N26910    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
H16757    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
N27732    CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC

          1651                                          1689
Bikunin   CTTTAG AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
H39841    CTTTAA.
N30199    CTTTAG.AAT AAA
T52966    CTTTAGGAAT NAAAANAAAA AAGGGTG
N29508    CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AA
N26919    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA A
N26910    CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AAAAAA
H16757    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAA
N27732    CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

FIGURE 4D

```
EST consens MLRAEADGVS RLLGSLLLSG VLAADRERSI HDFCLVSKVV GRCRASMPRW  50
EST consens WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATVTEN ATGDLATSRN 100
EST consens AADSSVPSAP RRQDSEDHSS DMFNYEEYCT ANAVTGPCRA SFPRWYFDVE 150
EST consens RNSCNNFIYG GCRGNKNSYR SEEACMLRCF RQQENPPLPL GSKVVVLAGL 200
EST consens FVMVLILFLG ASMVYLIRVA RRNQERALRT VWSSGDDKEQ LVKNTYVL   248
```

FIGURE 4E

```
cDNA                                                                ACC   3
translation                                                         T   -47

cDNA        TGATCGCGAG ACCCCAACGG CTGGTGGCGT CGCCTGCGCG TCTCGGCTGA  53
translation .  S  R  D   P  N  G   W  W  R   R  L  R  V   S  A  E -30

cDNA        GCTGGCCATG GCGCAGCTGT GCGGGCTGAG GCGGAGCCGG GCGTTTCTCG 103
translation  L  A  M   A  Q  L  C   G  L  R   R  S  R   A  F  L A-13

cDNA        CCCTGCTGGG ATCGCTGCTC CTCTCTGGGG TCCTGGCGGC CGACCGAGAA 153
translation   L  L  G   S  L  L   L  S  G  V   L  A  A   D  R  E   4

cDNA        CGCAGCATCC ACGACTTCTG CCTGGTGTCG AAGGTGGTGG GCAGATGCCG 203
translation R  S  I  H   D  F  C   L  V  S   K  V  V  G   R  C  R  21

cDNA        GGCCTCCATG CCTAGGTGGT GGTACAATGT CACTGACGGA TCCTGCCAGC 253
translation  A  S  M   P  R  W  W   Y  N  V   T  D  G   S  C  Q  L 38

cDNA        TGTTTGTGTA TGGGGGCTGT GACGGAAACA GCAATAATTA CCTGACCAAG 303
translation   F  V  Y   G  G  C   D  G  N  S   N  N  Y   L  T  K   54

cDNA        GAGGAGTGCC TCAAGAAATG TGCCACTGTC ACAGAGAATG CCACGGGTGA 353
translation E  E  C  L   K  K  C   A  T  V   T  E  N  A   T  G  D  71

cDNA        CCTGGCCACC AGCAGGAATG CAGCGGATTC CTCTGTCCCA AGTGCTCCCA 403
translation  L  A  T   S  R  N  A   A  D  S   S  V  P   S  A  P  R  88

cDNA        GAAGGCAGGA TTCTGAAGAC CACTCCAGCG ATATGTTCAA CTATGAAGAA 453
translation   R  Q  D   S  E  D   H  S  S  D   M  F  N   Y  E  E  104

cDNA        TACTGCACCG CCAACGCAGT CACTGGGCCT TGCCGTGCAT CCTTCCCACG 503
translation Y  C  T  A   N  A  V   T  G  P   C  R  A  S   F  P  R  121

cDNA        CTGGTACTTT GACGTGGAGA GGAACTCCTG CAATAACTTC ATCTATGGAG 553
translation  W  Y  F   D  V  E  R   N  S  C   N  N  F   I  Y  G  G 138

cDNA        GCTGCCGGGG CAATAAGAAC AGCTACCGCT CTGAGGAGGC CTGCATGCTC 603
translation   C  R  G   N  K  N   S  Y  R  S   E  E  A   C  M  L   154

cDNA        CGCTGCTTCC GCCAGCAGGA GAATCCTCCC CTGCCCCTTG GCTCAAAGGT 653
translation R  C  F  R   Q  Q  E   N  P  P   L  P  L  G   S  K  V 171

cDNA        GGTGGTTCTG GCGGGGCTGT TCGTGATGGT GTTGATCCTC TTCCTGGGAG 703
translation V  V  L  A   G  L  F   V  M  V   L  I  L   F  L  G  A 188

cDNA        CCTCCATGGT CTACCTGATC CGGGTGGCAC GGAGGAACCA GGAGCGTGCC 753
translation  S  M  V  Y   L  I   R  V  A  R   R  N  Q   E  R  A  204

cDNA        CTGCGCACCG TCTGGAGCTT CGGAGATGA                        782
translation L  R  T  V   W  S  F   G  D                           213
```

FIGURE 4F

```
cDNA         GCACGAGTTG GGAGGTGTAG CGCGGCTCTG AACGCGCTGA GGGCCGTTGA  50
cDNA         GTGTCGCAGG CGGCGAGGGC GCGAGTGAGG AGCAGACCCA GGCATCGCGC 100
cDNA         GCCGAGAAGG CCGGGCGTCC CCACACTGAA GGTCCGGAAA GGCGACTTCC 150
cDNA         GGGGGCTTTG GCACCTGGCG GACCCTCCCG GAGCGTCGGC ACCTGAACGC 200
cDNA         GAGGCGCTCC ATTGCGCGTG CGCGTTGAGG GGCTTCCCGC ACCTGATCGC 250
cDNA         GAGACCCCAA CGGCTGGTGG CGTCGCCTGC GCGTCTCGGC TGAGCTGGCC 300
cDNA         ATGGCGCAGC TGTGCGGGCT GAGGCGGAGC CGGGCGTTTC TCGCCCTGCT 350
translation  M  A  Q  L  C  G  L  R  R  S  R  A  F  L  A  L  L  -11

cDNA         GGGATCGCTG CTCCTCTCTG GGGTCCTGGC GGCCGACCGA GAACGCAGCA 400
translation  G  S  L  L  L  S  G  V  L  A  A  D  R  E  R  S  I   7

cDNA         TCCACGACTT CTGCCTGGTG TCGAAGGTGG TGGGCAGATG CCGGGCCTCC 450
translation     H  D  F  C  L  V  S  K  V  V  G  R  C  R  A  S   23

cDNA         ATGCCTAGGT GGTGGTACAA TGTCACTGAC GGATCCTGCC AGCTGTTTGT 500
translation  M  P  R  W  W  Y  N  V  T  D  G  S  C  Q  L  F  V   40

cDNA         GTATGGGGGC TGTGACGGAA ACAGCAATAA TTACCTGACC AAGGAGGAGT 550
translation  Y  G  G  C  D  G  N  S  N  N  Y  L  T  K  E  E  C   57

cDNA         GCCTCAAGAA ATGTGCCACT GTCACAGAGA ATGCCACGGG TGACCTGGCC 600
translation     L  K  K  C  A  T  V  T  E  N  A  T  G  D  L  A   73

cDNA         ACCAGCAGGA ATGCAGCGGA TTCCTCTGTC CCAAGTGCTC CCAGAAGGCA 650
translation  T  S  R  N  A  A  D  S  S  V  P  S  A  P  R  R  Q   90

cDNA         GGATTCTGAA GACCACTCCA GCGATATGTT CAACTATGAA GAATACTGCA 700
translation  D  S  E  D  H  S  S  D  M  F  N  Y  E  E  Y  C  T  107

cDNA         CCGCCAACGC AGTCACTGGG CCTTGCCGTG CATCCTTCCC ACGCTGGTAC 750
translation     A  N  A  V  T  G  P  C  R  A  S  F  P  R  W  Y   123

cDNA         TTTGACGTGG AGAGGAACTC CTGCAATAAC TTCATCTATG GAGGCTGCCG 800
translation  F  D  V  E  R  N  S  C  N  N  F  I  Y  G  G  C  R  140

cDNA         GGGCAATAAG AACAGCTACC GCTCTGAGGA GGCCTGCATG CTCCGCTGCT 850
translation  G  N  K  N  S  Y  R  S  E  E  A  C  M  L  R  C  F  157

cDNA         TCCGCCAGCA GGAGAATCCT CCCCTGCCCC TTGGCTCAAA GGTGGTGGTT 900
translation     R  Q  Q  E  N  P  P  L  P  L  G  S  K  V  V  V   173

cDNA         CTGGCGGGGC TGTTCGTGAT GGTGTTGATC CTCTTCCTGG GAGCCTCCAT 950
translation  L  A  G  L  F  V  M  V  L  I  L  F  L  G  A  S  M  190

cDNA         GGTCTACCTG ATCCGGGTGG CACGGAGGAA CCAGGAGCGT GCCCTGCGCA 1000
translation  V  Y  L  I  R  V  A  R  R  N  Q  E  R  A  L  R  T  207

cDNA         CCGTCTGGAG CTCCGGAGAT GACAAGGAGC AGCTGGTGAA GAACACATAT 1050
translation     V  W  S  S  G  D  D  K  E  Q  L  V  K  N  T  Y   223

cDNA         GTCCTGTGAC CGCCCTGTCG CCAAGAGGAC TGGGGAAGGG AGGGGAGACT 1100
translation  V  L  *                                              225
```

FIGURE 4F (Con't)

```
cDNA        ATGTGTGAGC TTTTTTTAAA TAGAGGGATT GACTCGGATT TGAGTGATCA 1150
cDNA        TTAGGGCTGA GGTCTGTTTC TCTGGGAGGT AGGACGGCTG CTTCCTGGTC 1200
cDNA        TGGCAGGGAT GGGTTTGCTT TGGAAATCCT CTAGGAGGCT CCTCCTCGCA 1250
cDNA        TGGCCTGCAG TCTGGCAGCA GCCCCGAGTT GTTTCCTCGC TGATCGATTT 1300
cDNA        CTTTCCTCCA GGTAGAGTTT TCTTTGCTTA TGTTGAATTC CATTGCCTCC 1350
cDNA        TTTTCTCNAT CACAGAAGTG ATGTTGGAAT CGTTTCTTTT GTTTGTCTGA 1400
cDNA        TTTATGGTTT TTTTAAGTAT AAACAAAAGT TTTTTATTAG CATTCTGAAA 1450
cDNA        GAAGGAAAGT AAAATGTACA AGTTTAATAA AAAGGGGCCT TCCCCTTTAG 1500
cDNA        AATAAATTTC CAGCATGTTG CTTTCAAAAA AAAAAAAAAA AAAA
1550
```

FIGURE 4G

```
EST consens                                    MLR AEADGVSRLL GSLLLSGVLA  -1
PCR clone                                    MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
λcDNA clone                                  MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

EST consens ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
PCR clone   ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
λcDNA clone ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

EST consens YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
PCR clone   YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
λcDNA clone YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

EST consens NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
PCR clone   NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
λcDNA clone NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

EST consens ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
PCR clone   ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
λcDNA clone ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

EST consens QERALRTVWS SGDDKEQLVK NTYVL                           225
PCR clone   QERALRTVWS FGD                                       213
λcDNA clone QERALRTVWS SGDDKEQLVK NTYVL                           225
```

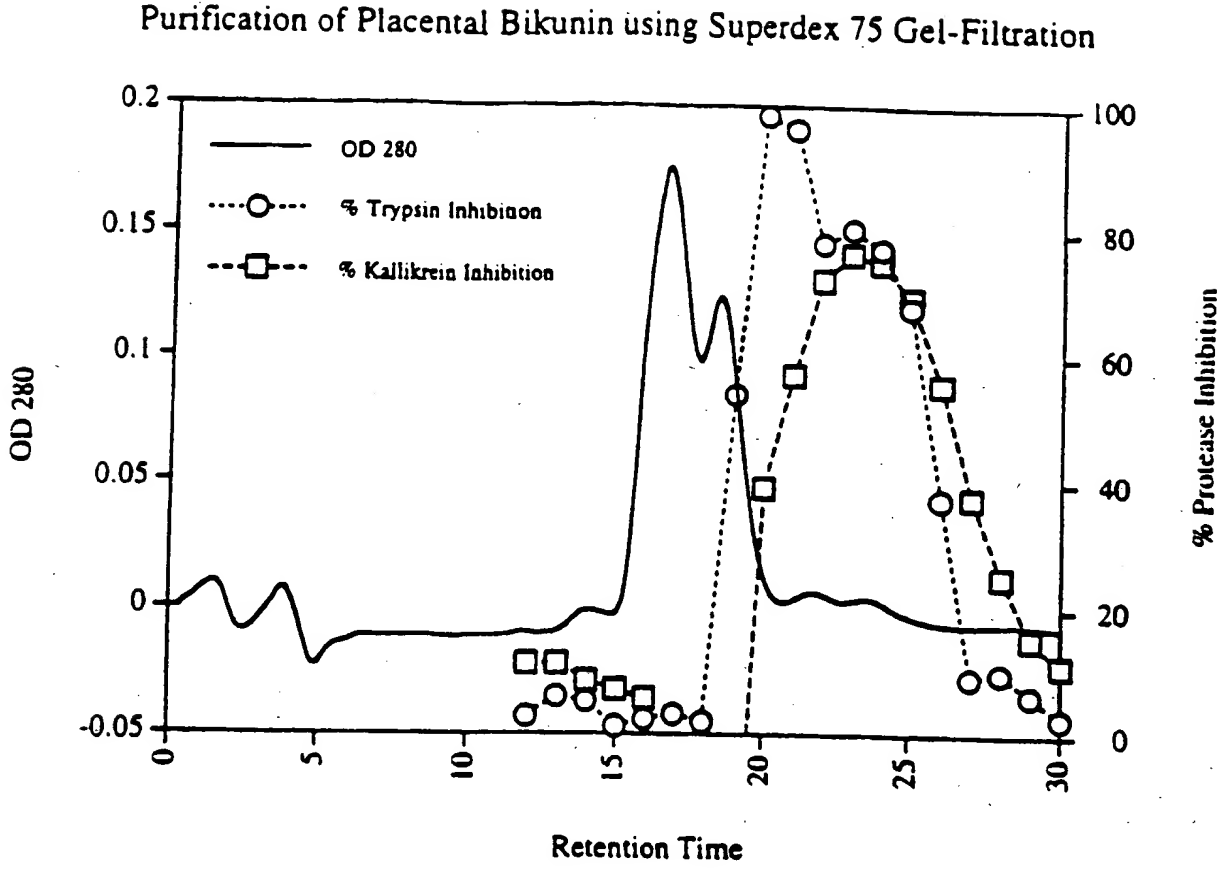
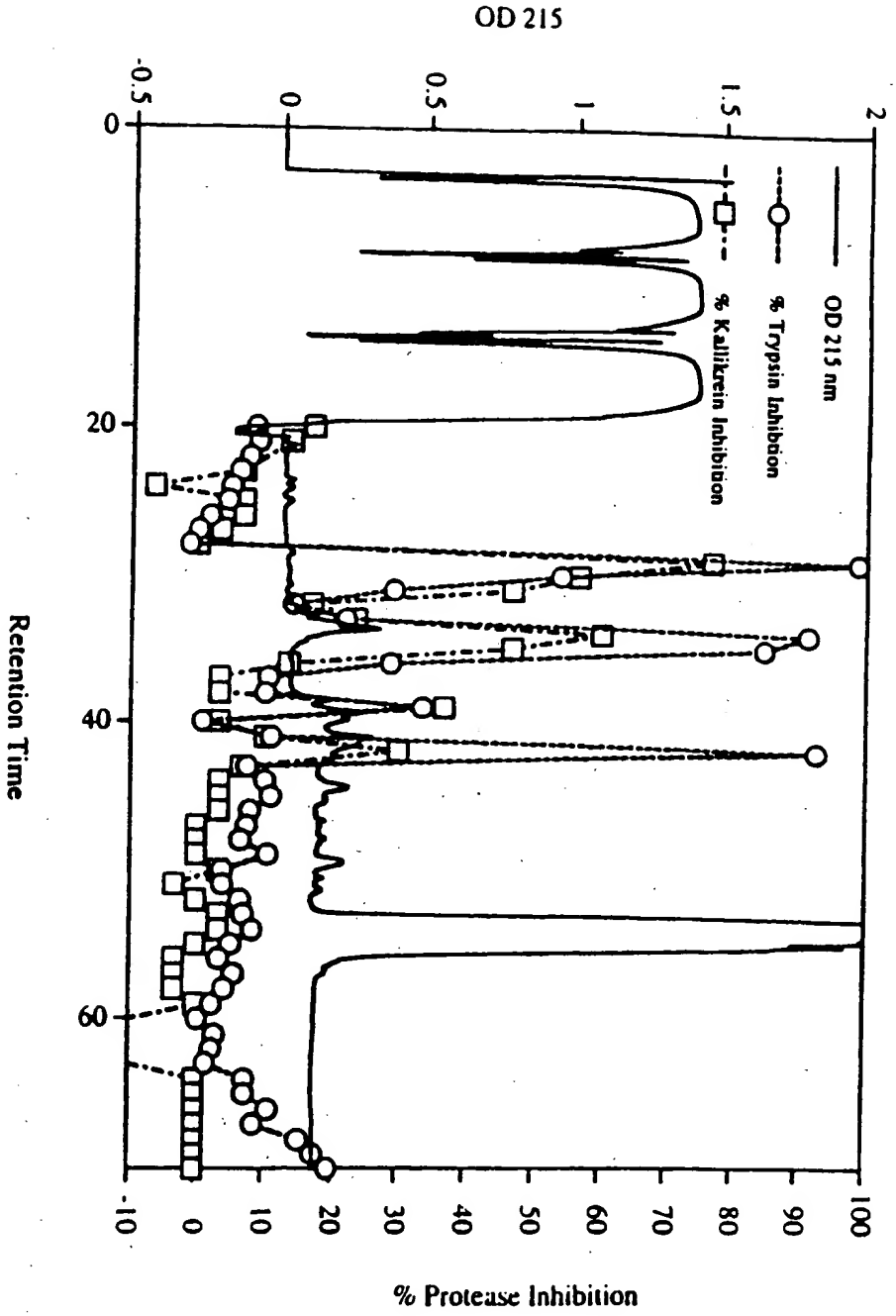

Purification of Placental Bikunin using Superdex 75 Gel-Filtration
OD 280
% Trypsin Inhibition
% Kallikrein Inhibition
OD 280
% Protease Inhibition
Retention Time
0.2
0.15
0.1
0.05
0
-0.05
100
80
60
40
20
0
0
5
10
15
20
25
30

FIGURE 5



Purification of Placental Bikunin using C18 Reverse-Phase Chromatography

FIGURE 6

Figure 7

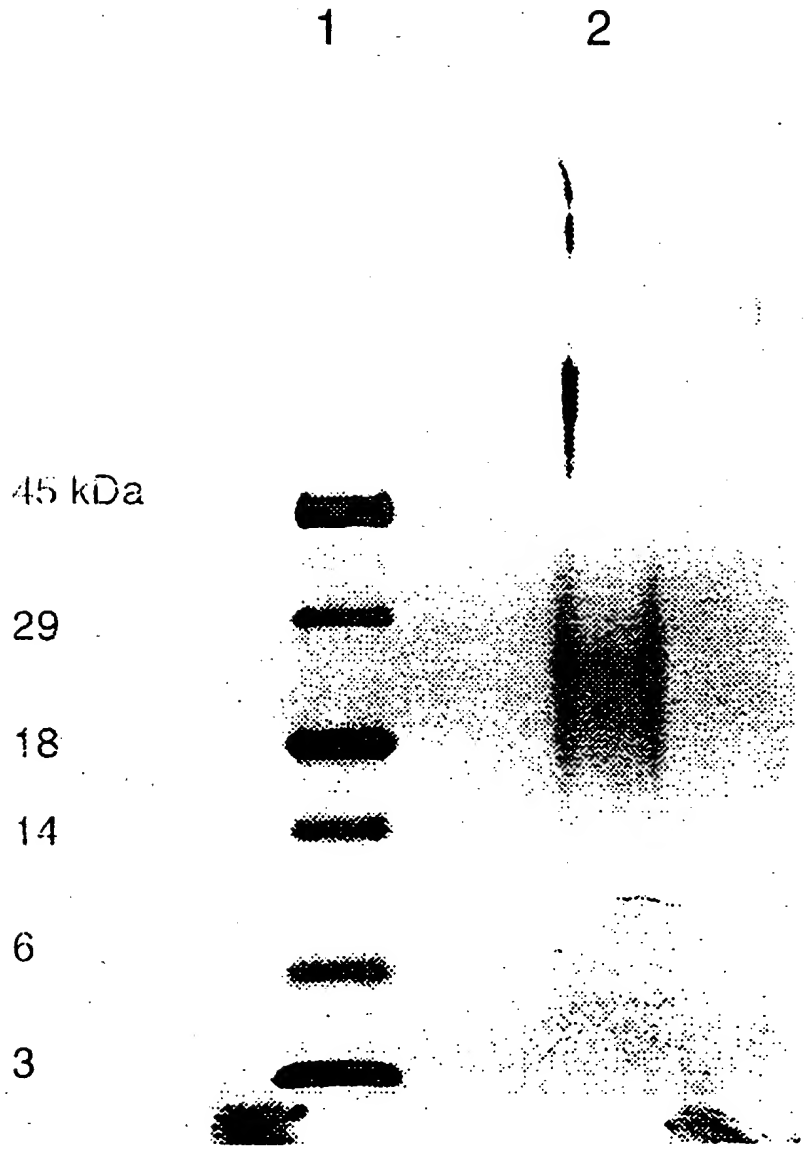


Figure 8A

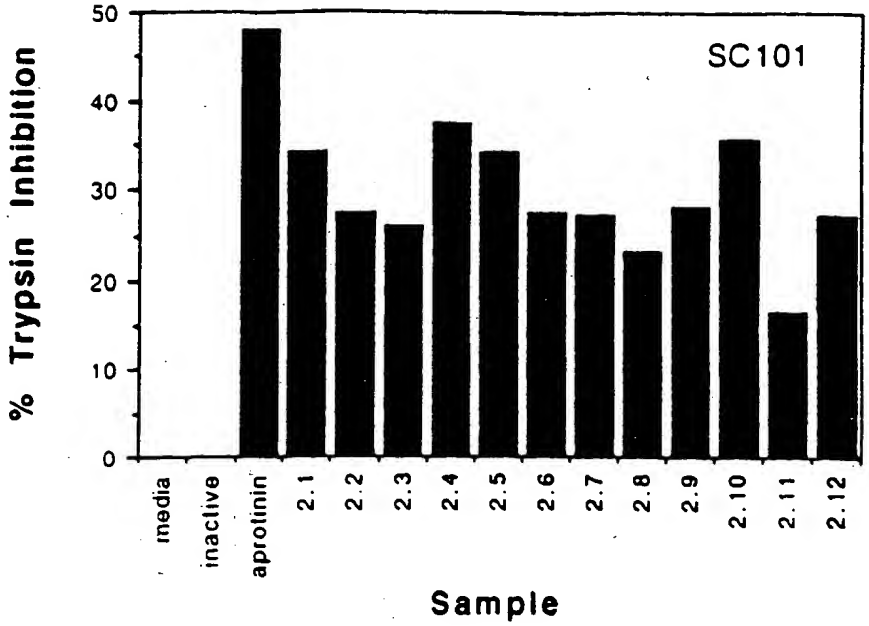


Figure 8B

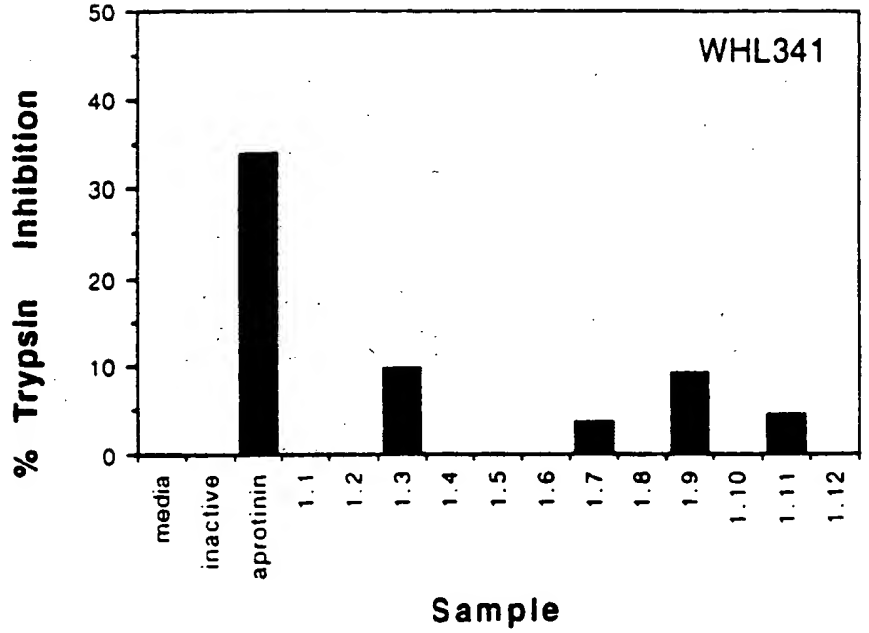


Figure 9A
SDS-PAGE
Aprotinin
2.5
2.4
45 kDa
29
18
14
6
3

Figure 9B
Western
Aprotinin
2.5
2.4
45 kDa
29
18
14
6
3

Figure 12B

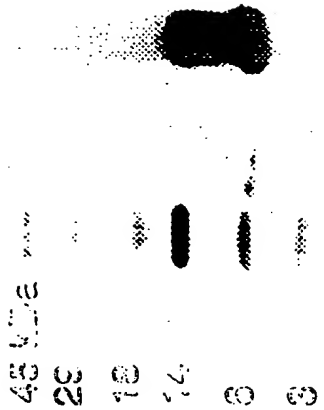


Figure 12A

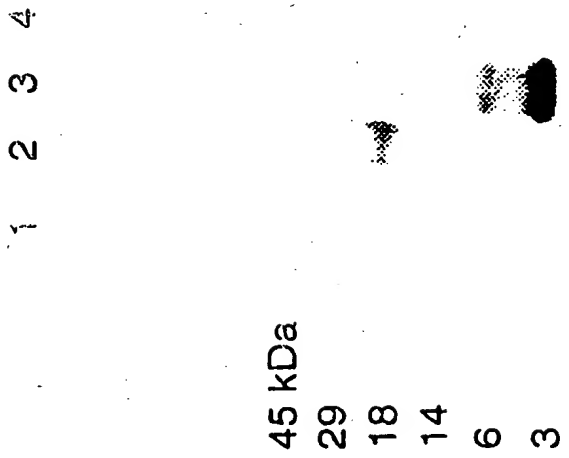


Figure 13

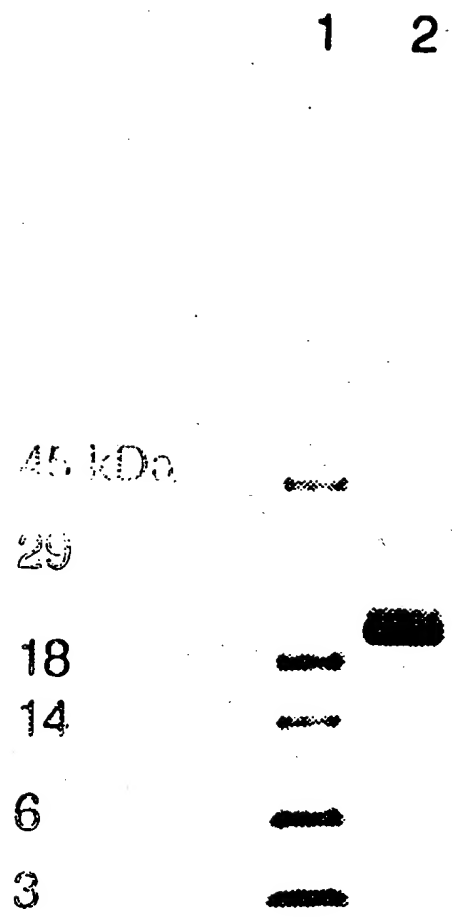


Figure 10

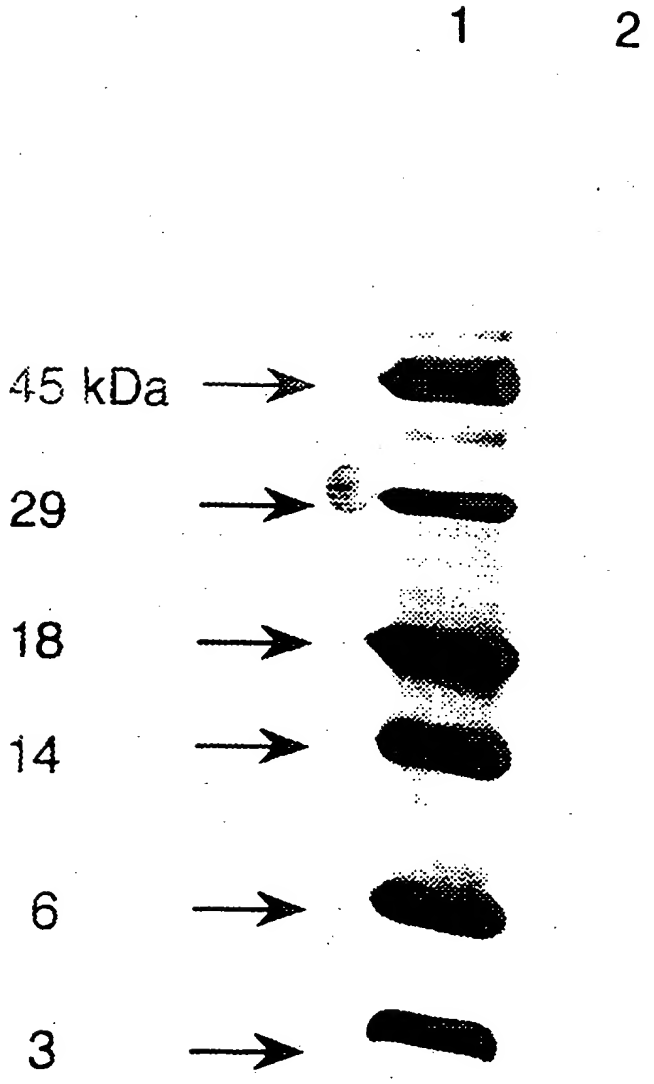


Figure 11B

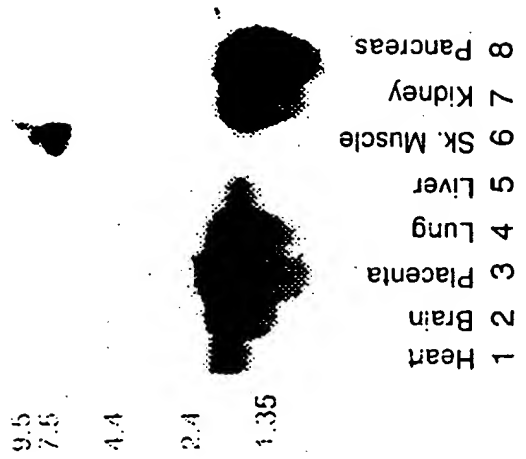


Figure 11A

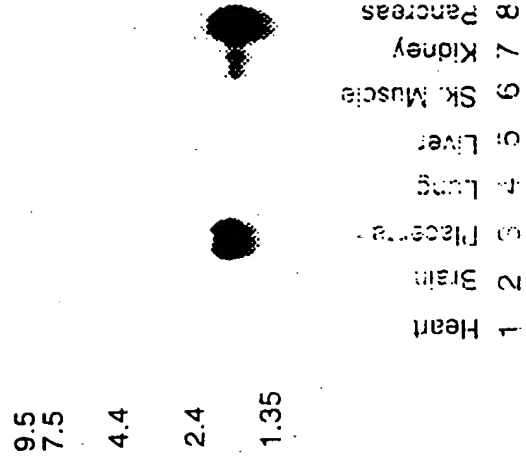
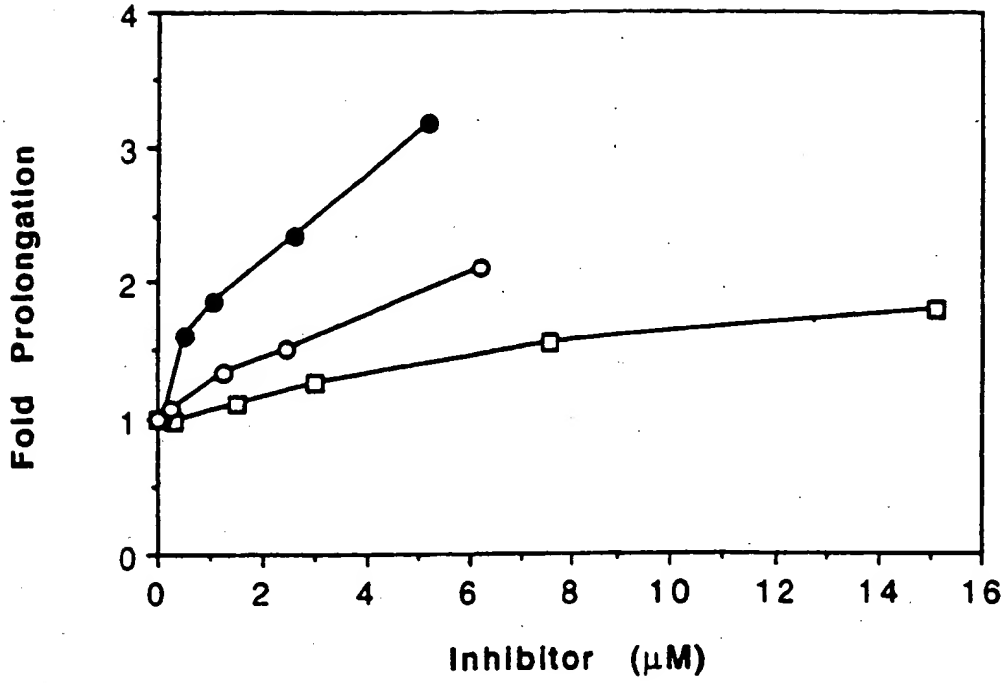


Figure 14



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/03894

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/81 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R35464 Sequence reference HS46499, May 4, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039653 see the whole document ---	1-6,11
X	EMBL/GENBANK DATABASES Accession no N39798 Sequence reference HS798277, January 26, 1996 L. HILLIER ET AL: "The WasU-Merck EST Project" XP002039654 see the whole document --- -/--	1-6,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
4 September 1997	0 1. 10. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk	Authorized officer

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 97/03894

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R74593 Sequence reference HS593137,June 9, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039655 see the whole document	1-6,11
P,X	EP 0 758 682 A (MITSUBISHI CHEM CORP) 19 February 1997 see the whole document	1-11
P,A	JOURNAL OF BIOLOGICAL CHEMISTRY 272 (10). 1997. 6370-6376. ISSN: 0021-9258, XP002039700 SHIMOMURA T ET AL: "Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor."	
A	DATABASE MEDLINE accession no 94289695, 1 July 1994 J. WOJTA ET AL: "Hepatocyte growth factor stimulates expression of plasminogen activator inhibitor type 1 and tissue factor in HepG2 cells" XP002039702 see abstract & BLOOD, vol. 84, no. 1, 1994, pages 151-157,	7-10
A	JOURNAL OF BIOLOGICAL CHEMISTRY 271 (7). 1996. 3615-3618. ISSN: 0021-9258, XP002039701 MIYAZAWA K ET AL: "Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator."	7-10

INTERNATIONAL SEARCH REPORT

Int' ~ational application No

PCT/US 97/03894

B x I Observations where certain claims were found unsearchable (Continuati n of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 7-9
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter al Application No
PCT/US 97/03894

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0758682 A	19-02-97	JP 9095498 A	08-04-97